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(54) **Hybrid molecules having translocation region and cell-binding region.**

(57) A hybrid molecule including a first part, a second part and a third part connected by covalent bonds,
(a) the first part including a portion of the binding domain of a cell-binding ligand, which portion is able to cause the hybrid molecule of the invention to bind to an animal cell ;
(b) the second part including a portion of a translocation domain of a protein, which portion is capable of translocating the third part across the cytoplasmic membrane of the cell ; and
(c) the third part including a chemical entity to be introduced into the cell, provided that (i) the hybrid molecule is produced by expression of a recombinant DNA molecule encoding the hybrid molecule, and (ii) the second and third parts are not segments of the same naturally-occurring polypeptide toxin ;
a recombinant DNA molecule encoding the hybrid molecule ; and
a method of producing the hybrid molecule.

EP 0 439 954 A2

HYBRID MOLECULES HAVING TRANSLOCATION REGION AND CELL-BINDING REGION

Background of the Invention

This invention relates to hybrid molecules having a cell-binding part and a translocation part.

The literature contains many examples of fused genes which code for hybrid proteins. For example, Villal-Komaroff et al., Proc. Natl. Acad. Sci. U.S.A. 75 : 3727-3731, 1978, describes a fused gene made up of a eukaryotic structural gene fused to a non-cytoplasmic bacterial gene. The fused gene codes for a hybrid protein which is transported out of the cytoplasm.

Hybrid proteins also have been made by other methods (e.g., the coupling of two different protein molecules) which do not involve recombinant DNA techniques. For example, it has been proposed to form, by coupling, therapeutic hybrid proteins consisting of portions of toxin molecules coupled to a ligand capable of binding specifically to a selected class of cells. One attempt to make such a hybrid protein, reported in Chang et al., J. Biol. Chem. 252 :1515-1522, 1977, resulted in a hybrid consisting of the diphtheria toxin A chain coupled to human placental lactogen hormone by cross-linking through a disulfide bond. Although the hybrid protein bound to cells containing lactogen receptors, it did not inhibit protein synthesis in those cells.

A hybrid protein consisting of the ricin toxin A chain coupled to the β chain of human chorionic gonadotropin hormone by similarly cross-linking through a disulfide bond has also been reported ; although said to have specificity, its binding capacity has not been reported. Furthermore, extremely high concentrations were required to significantly inhibit protein synthesis in rat Leydig tumor cells, making it difficult to distinguish between "non-specific" entry caused by endocytosis and "specific" entry caused by transport of the toxic portion of the hybrid across the cytoplasmic membrane of the target cells (Oeltman et al., J. Biol. Chem. 254 :1028-1032, 1979). The same shortcoming was found in a hybrid protein consisting of diphtheria A coupled to insulin using cystamine as the cross-linking agent (Miskimins et al., Biochem. Biophys. Res. Commun. 91 :143-151, 1979). A hybrid consisting of ricin A coupled to epidermal growth factor (EGF) by means of a heterobifunctional cross-linker has also been made ; the binding characteristics provided by the EGF are not limited to specific cells, but rather encompass a wide variety of cell types (Cawley et al., Cell 22 :563-570, 1980).

As illustrated in Fig. 1, the natural diphtheria toxin molecule consists of several functional "domains" which can be characterized, starting at the amino terminal end of the molecule, as hydrophobic leader signal sequence s (amino acids Val₂₅ - Ala₁) ; enzymatically-active Fragment A (amino acids Gly₁ - Arg₁₉₃) ; the protease-sensitive disulfide loop I₁ (amino acids Cys₁₈₈ - Cys₂₀₁), containing a cleavage domain ; and Fragment B (amino acids Ser₁₉₄ - Ser₅₃₅), which includes a translocation domain and a generalized binding domain flanking a second disulfide loop (I₂, amino acids Cys₄₆₁ - Cys₄₇₁).

The process by which diphtheria toxin intoxicates sensitive eukaryotic cells involves at least the following steps : (i) the binding domain of diphtheria toxin binds to specific receptors on the surface of a sensitive cell ; (ii) while bound to its receptor, the toxin molecule is internalized into an endocytic vesicle ; (iii) either prior to internalization, or within the endocytic vesicle, the toxin molecule undergoes a proteolytic cleavage in I₁ between fragments A and B ; (iv) as the pH of the endocytic vesicle decreases to below 6, the toxin spontaneously inserts into the endosomal membrane ; (v) once embedded in the membrane, the translocation domain of the toxin facilitates the delivery of Fragment A into the cytosol ; (vi) the catalytic activity of Fragment A (i.e., the nicotinamide adenine dinucleotide - dependent adenosine diphosphate (ADP) ribosylation of the eukaryotic protein synthesis factor termed "Elongation Factor 2") causes the death of the intoxicated cell. It is apparent that a single molecule of Fragment A introduced into the cytosol is sufficient to shut down the cell's protein synthesis machinery and kill the cell. The mechanism of cell killing by *Pseudomonas* exotoxin A, and possibly by certain other naturally-occurring toxins, is very similar.

Summary of the Invention

In general, the invention features, in one aspect, a hybrid molecule including a first part, a second part, and a third part connected by covalent bonds,

(a) the first part including a portion of the binding domain of a cell-binding ligand, which portion is able to cause the hybrid molecule of the invention to bind to an animal cell ;

(b) the second part including a portion of a translocation domain of a protein, which portion is capable of translocating the third part across the cytoplasmic membrane of the cell ; and

(c) the third part including a chemical entity (here, a polypeptide) to be introduced into the cell, provided that (i) the hybrid molecule is produced by expression of a recombinant DNA molecule encoding the hybrid molecule, and (ii) the second and third parts are not segments of the same naturally-occurring polypeptide

toxin, (although the first and second parts may be derived from the same toxin : e.g., diphtheria toxin or *Pseudomonas* exotoxin A). "Translocation" here means the facilitation of movement of a chemical entity from the exterior surface of a cellular membrane (or what constituted the exterior surface prior to formation of an endocytic vesicle), through the membrane, and into the cytosol at the interior of the cell. A "translocation domain" is a segment of a protein which, when the protein is bound to the exterior surface of a cellular membrane, is capable of translocating some portion of that protein through the membrane.

The invention also features a hybrid molecule including a first part, a second part, and a third part connected by covalent bonds,

(a) the first part including a portion of the binding domain of a cell-binding ligand (which may be a polypeptide ligand or may be another type of ligand such as a steroid hormone), which portion is effective to cause the hybrid molecule to bind to a cell of an animal ;

(b) the second part including a portion of a translocation domain of a protein, which portion is capable of translocating the third part across the cytoplasmic membrane of the cell ; and

(c) the third part including a chemical entity (which may or may not be a polypeptide) to be introduced into the cell, provided that (i) the first and second parts are not segments of the same naturally-occurring polypeptide toxin, and (ii) likewise, the second and third parts are not segments of the same naturally-occurring polypeptide toxin.

In preferred embodiments, the second part of either type of hybrid molecule described above comprises at least a portion of the translocation domain of a naturally-occurring toxin (e.g. diphtheria toxin or *Pseudomonas* exotoxin A), and the ligand comprises a hormone (e.g., a polypeptide hormone such as insulin, Interleukin II (also termed "IL2"), Interleukin IV, Interleukin VI or EGF) ; an antigen-binding, single-chain analog of a monoclonal antibody ; or a polypeptide toxin capable of binding to the desired class of cells. Where all three parts are polypeptides, the hybrid molecule is preferably a recombinant protein (that is, a protein produced by recombinant DNA techniques). More preferably, the third part is an antigen-binding, single-chain analog of a monoclonal antibody (where such antigen is, for example, a viral protein such as the human immunodeficiency virus (HIV) protease), or alternatively, the enzymatically active portion of an enzyme (e.g., hexosaminidase A ; α -1,4-glucosidase ; phenylalanine hydroxylase ; a protease ; a nuclease ; or a toxin such as cholera toxin, LT toxin, C3 toxin, Shiga toxin, *E.coli* Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, diphtheria toxin or *Pseudomonas* exotoxin A), and most preferably it supplies an enzymatic activity in which the cell is deficient : as, for example, in the case of a genetic deficiency. Where the enzyme is cholera toxin, the resulting hybrid molecule may be used to raise the cyclic AMP level within a target animal cell : preferably, the target cell so treated is a T-cell and the hybrid molecule includes at least a portion of the binding domain of IL2. The hybrid molecule of the invention includes the cholera toxin A/diphtheria toxin B'/IL2 hybrid polypeptide encoded by the plasmid illustrated in Fig. 6 ; the Shiga-like toxin A/diphtheria toxin B'/ IL2 hybrid polypeptide encoded by the plasmid illustrated in Fig. 9 ; the ricin A/diphtheria toxin B'/IL2 hybrid polypeptide encoded by the plasmid illustrated in Fig. 12 ; the phenylalanine hydroxylase/diphtheria toxin fragment B hybrid polypeptide encoded by the plasmid illustrated in Fig. 14 ; an HIV protease-binding protein (HIVP-BP)/diphtheria toxin B'/IL2 hybrid polypeptide prepared as hereinafter described ; and a Shiga-like toxin A/IL2 hybrid in which both the enzymatic activity and the translocation function are provided by the Shiga-like toxin A portion of the hybrid, and which contains a proteolytically-sensitive disulfide loop. Also included are biologically active mutational analogs of any of the above hybrid polypeptides. As used herein, a "biologically active mutational analog" is a polypeptide which exhibits the same type of cell-binding specificity and the same type of biological activity (e.g., a particular enzymatic or antigen-binding activity) as the listed hybrid polypeptide of which it is an analog, but which differs from such listed hybrid polypeptide by one or more deletions and/or one or more substitutions of segments of one or more amino acid residues. Preferably, the amino acid sequence of the biologically active mutational analog shows at least a 70% (more preferably 80% and most preferably 90%) homology (i.e., identity of amino acid sequence) with the hybrid polypeptide of which it is an analog, and the analog exhibits at least 50% (more preferably, at least 75%) of a biological activity exhibited by the hybrid polypeptide of which it is an analog.

Also within the invention is a recombinant DNA molecule encoding any of the above hybrid polypeptide molecules (including biologically active mutational analogs), a vector including such a recombinant DNA molecule, a cell containing such a vector or recombinant DNA molecule (and which preferably is capable of expressing the recombinant DNA molecule to produce the hybrid polypeptide encoded by it), and a method of preparing the hybrid polypeptide molecule of the invention by permitting a cell containing a recombinant DNA molecule encoding the polypeptide (the "transformed cell") to express the recombinant DNA molecule.

In other preferred embodiments, the third part comprises a detectable label, more preferably a fluorescent moiety, a radioactive moiety, or an electron-dense moiety.

The invention also features a method of labeling a class of cells, which method involves contacting the cells with a hybrid molecule having a third part comprising a detectable label.

Also included in the invention are (1) a method of treating an animal having a deficiency in a certain enzyme, by administering to the animal an effective amount of a hybrid molecule comprising that enzyme ; and (2) a method of treating a human patient infected with HIV, by administering to the patient an effective amount of a hybrid molecule having as its third part an HIV protease-binding, single-chain analog of a monoclonal antibody against HIV protease.

Based upon the observation that certain types of polypeptide toxins have three separate functional regions, one region which binds the molecule to particular receptors on the surface of a target cell, a second one which facilitates entry of the enzymatically-active region into the cytosol of the cell, and a third region which exhibits the enzymatic activity that characterizes the toxic effect of the molecule, the invention includes tripartite hybrid molecules in which any of these regions may be replaced with functionally comparable regions from other sources. That is, the first functional region may be replaced with a particular binding moiety which binds the hybrid molecule to a selected class of cells, such as IL2 (which binds to high-affinity IL2 receptor-bearing T-cells), or α melanocyte stimulating hormone (α MSH, which binds to melanocytes), or a moiety which binds to a broad spectrum of cell types, as is characteristic of the binding domains of cholera toxin and diphtheria toxin ; the second part may be taken from any type of polypeptide in which a translocation domain is identifiable, but will most likely be from a toxin molecule that translocates in a manner similar to diphtheria toxin and *Pseudomonas* exotoxin A. The third part may be any type of moiety that one wants to insert into the cell and that will fit through the channel in the membrane formed by the translocation domain : for example, a cell-killing enzyme such as Shiga toxin ; a metabolic enzyme such as phenylalanine hydroxylase (the enzyme in which phenylketonurics are deficient) ; an antigen-binding, single-chain analog of a monoclonal antibody against an antigen that appears within the target cell ; or a fluorescent label. A proteolytically-sensitive loop (such as I₁ of diphtheria toxin) between the enzymatically-active region of the hybrid and the remainder of the hybrid appears to play an important role in the hybrid's function.

Although the medical community is rapidly expanding its understanding of the molecular bases of many diseases, one problem has particularly frustrated efforts to translate this understanding into rational protocols for treating the diseases : the problem of how to direct the appropriate therapy into the affected cells so that it can function properly to alleviate or cure the disease. By providing such a method, the present invention will have virtually unlimited applications : from treating genetic deficiency diseases by delivering to affected cells an enzyme supplying the missing function, to supplementing cellular levels of a particular enzyme or a scarce precursor or cofactor, to directing toxins or other poisons to destroy particular cells (such as adipocytes, cancer cells, or virus-infected cells), to counteracting viral infections such as HIV (which causes Acquired Immunodeficiency Syndrome ("AIDS")) by introducing into appropriate cells antibodies to viral proteins. The invention also provides a means for getting other, non-therapeutic substances, such as detectable labels, into targeted cells. The use of a translocation mechanism ensures that the hybrid will be effective in relatively low doses, since a high proportion of the substance of interest will be taken into the targeted cells.

To the extent that the three parts of the hybrids of the invention are polypeptides, they may be manufactured as a single hybrid recombinant protein, permitting reproducibility, consistency, and the precise control of composition which is desirable for any pharmaceutical product.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Description of the Preferred Embodiments

The drawings will be briefly described.

Drawings

Fig. 1 is a diagrammatic representation of the diphtheria toxin molecule.

Fig. 2 is a restriction map showing the location and orientation of the diphtheria tox gene on the 3.9 kb BamHI restriction fragment of corynebacterium β ^{tox}.

Fig. 3 is a representation of the diphtheria toxin gene and flanking regions, with the protein encoded shown above ; the B' region is the region between the labeled Sau3A1-2 and SphI sites.

Fig. 4 is a diagrammatic representation of the strategy used to construct a plasmid encoding fragment B' of diphtheria toxin.

Fig. 5 is a representation of the nucleotide sequence of the Vibrio cholerae toxin gene, with amino acids shown below corresponding codons.

Fig. 6 is a diagrammatic representation of the cloning strategy followed to construct a plasmid encoding cholera toxin A₁-diphtheria toxin B'- IL2 hybrid.

Fig. 7 is a diagrammatic representation of the cloning strategy followed in order to construct the plasmid pPA123.

Fig. 8 is a representation of the nucleotide sequence of the *E.coli* bacteriophage H19B Shiga-like toxin gene, with amino acids shown below corresponding codons.

Fig. 9 is a diagrammatic representation of a cloning strategy proposed for constructing a plasmid encoding a Shiga-like toxin A-diphtheria toxin B'-IL2 hybrid.

Fig. 10 is a diagrammatic representation of an alternative cloning strategy for constructing a plasmid encoding a Shiga-like toxin A-diphtheria toxin B'-IL2 hybrid.

Fig. 11 is a representation of the nucleotide sequence of the *Ricinus communis* ricin gene, with amino acids shown above corresponding codons; this figure is adapted from Fig. 2 of Halling et al. (Nucl. Acids Res. 13 :8019-8033, 1985).

Fig. 12 is a diagrammatic representation of the cloning strategy proposed for constructing a plasmid encoding a ricin A-diphtheria toxin B'-IL2 hybrid.

Fig. 13 is a representation of the nucleotide sequence of human phenylalanine hydroxylase cDNA, with amino acids shown below corresponding codons.

Fig. 14 is a diagrammatic representation of a cloning strategy proposed for constructing a plasmid encoding a phenylalanine hydroxylase-diphtheria toxin B hybrid.

Structure

One embodiment of the hybrid molecule of the invention is a three-part hybrid protein which includes (1) a cell-binding domain; (2) a translocation domain, such as that of diphtheria toxin; and (3) a polypeptide to be introduced into the cell, which polypeptide is not derived from the same naturally-occurring protein as is the translocation domain sequence. The cell-binding domain may be generalized (i.e., it is capable of binding the hybrid molecule to a wide variety of cell types, like the cell-binding domain of naturally-occurring diphtheria toxin), or specific for one or a few types of cells. The third part of the hybrid is covalently linked to the translocation domain, such that the translocation domain is capable of translocating the third part into or across the membrane of the cell to which the cell-binding portion of the hybrid is bound. This third part may be, for example, an enzymatically active polypeptide, an antigen-binding portion of a monoclonal antibody, or a detectable label such as a fluorescent dye. It may not, however, be a fragment of the same naturally-occurring molecule from which the translocation domain originates.

Naturally-occurring proteins which are known to have a translocation domain include diphtheria toxin and *Pseudomonas* exotoxin A, and may include other toxins and non-toxin molecules, as well. The translocation domains of diphtheria toxin and *Pseudomonas* exotoxin A are well characterized (see, e.g., Hoch et al., Proc. Natl. Acad. Sci. USA 82 :1692-1696, 1985; Colombatti et al., J. Biol. Chem. 261 :3030-3035, 1986; and Deleers et al., FEBS 160 :82-86, 1983), and the existence and location of such a domain in other molecules may be determined by methods such as those employed by Hwang et al., Cell 48 :129-136, 1987; and Gray et al., Proc. Natl. Acad. Sci. USA 81 :2645-2649, 1984.

The segment of diphtheria toxin labelled "Fragment B" in Fig. 3 includes both the translocation domain and the generalized cell-binding domain of the naturally-occurring molecule. Truncation of Fragment B to the segment marked B' effectively eliminates the cell-binding function of diphtheria toxin while retaining the translocation function of the molecule. A portion of Fragment B encoded by a sequence ending at or downstream from the *Sph*I restriction site may be used for the translocation domain; however, if the hybrid includes a cell-binding region other than that of diphtheria toxin, the portion of Fragment B downstream from the *Sph*I site may be included only if it does not include sequences encoding a sufficient part of the diphtheria toxin receptor-binding domain to yield a functional diphtheria toxin receptor-binding domain.

The part of the hybrid protein contributed by the polypeptide ligand can consist of the entire ligand, or a portion of the ligand which includes the entire binding domain of the ligand, or an effective portion of the binding domain. When the ligand being used is large, it is desirable that as little of the non-binding portion as possible of the ligand be included, so that the binding domain of the molecule is positioned close to the translocation domain. It is also desirable to include all or most of the binding domain of the ligand molecule.

The polypeptide portions of the hybrids of the invention are conveniently made using recombinant DNA techniques involving forming the desired fused gene encoding the hybrid protein, and then expressing the fused gene. Chemical cross-linking is utilized only where one or more of the parts of the hybrid molecule are not polypeptides.

Standard procedures for DNA cloning, cell transformation and plasmid isolation (as described, for example, by Maniatis et al., Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), and for oligodeoxynucleotide synthesis, could be employed to carry out the following construc-

tions.

Example 1. Fusion of a gene fragment encoding diphtheria toxin B' with sequence encoding cell-binding portions of various ligands ; labeling and use of the resultant hybrid polypeptides

Referring to Figs. 2 and 3, the location and orientation of the diphtheria tox operon on the 3.9 kb BamHI restriction fragment of corynebacteriophage β^{tox} allows the tox operon to be cleaved at a desired location, and the desired portion of the operon to be fused with the desired portion of the gene for a selected polypeptide ligand.

Gene fusions of the invention may be made as follows. First, the NsiI-SphI fragment encoding most of Fragment B' (Fig. 3) is isolated from the tox gene of plasmid pABM508 (Murphy et al., Proc. Natl. Acad. Sci. USA 83 :8258-8262, 1986). The following linker is ligated into the 5' (NsiI) end :

```

5'      C ATG TCA GTA GGT AGC TCA TTG TCA TGC A   3'
3'      AGT CAT CCA TCG AGT AAC AGT               5'
encoding fmet-Ser-Val-Gly-Ser-Ser-Leu-Ser-Cys
          1/2                               1/2
          NcoI                             NsiI

```

and the following linker is ligated into the 3' (SphI) end :

```

5'      CAT GAA   3'
3'      G TAC GTA CTT TCG A   5'
          1/2               1/2
          SphI             HindIII

```

The resulting fragment is then cloned into NcoI + HindIII-digested pKK233-2 (Pharmacia, Piscataway, NJ).

This modification allows the expression of Fragment B' to be driven off the trc promoter (P_{trc}) in E.coli. The SphI site permits in-frame fusion with a gene sequence encoding the binding domain of a peptide ligand.

Generally, the manipulative operations are carried out using cloning vectors ; e.g., phages or plasmids. The genetic material coding for the binding domain of the polypeptide ligand can be either DNA cloned from a natural source, or a synthetic oligonucleotide sequence. Generally the fused gene will reside on a cloning vector, e.g., a plasmid or a phage, which is used to transform cultured bacteria, yeast or tissue culture host cells. The hybrid protein is then harvested from the cells using conventional techniques. Purification of the hybrid proteins of the invention, regardless of the polypeptide ligand used, can be carried out via affinity chromatography, using a monoclonal antibody against diphtheria toxin Fragment B.

The purified hybrid protein of the invention may be used as a transport system to carry a detectable label into specific cells. The label which is attached to the hybrid protein molecule can be any conventional atom or molecule used for diagnostic labeling ; examples are radioactive labels such as 125 I-compounds, technetium isotopes, NMR reporter groups, and fluorescent dyes. The most preferred labels are hydrophobic labels such as fluorescent dyes (most conventional fluorescent dyes happen to be hydrophobic) which are incorporated into the cytoplasmic membrane of target cells, as will be explained in more detail below. Labels can be attached to the hybrid protein according to conventional labeling techniques. Labels are used in an amount (e.g., one or two label molecules per protein molecule) which does not interfere with the binding or cell penetration functions of the hybrid protein molecule.

The labeled hybrid proteins of the invention can be used diagnostically to label a desired class of target cells, a class determined by the specific polypeptide ligand which imparts the binding domain of the hybrid molecule. The specific binding domain of the polypeptide ligand portion selectively binds to those cells ; the labeled molecule is then taken up by the cells via receptor-mediated endocytosis, and the label subsequently is delivered to the cell membrane and/or the cytoplasm of the target cells.

The process by which a labeled hybrid protein of the invention can be incorporated into cells can be summarized as follows. The labeled hybrid protein is taken up by target cells via receptor-mediated endocytosis into an endocytic vesicle ; thereafter a pH differential across the membrane of the endocytic vesicle is established as a result of the cell's ATP-dependent proton pump. The pH differential across the membrane causes the hybrid protein, including its lipid-associating portion and its label, to be inserted into the plane of the membrane of the endocytic vesicle. The hydrophobic nature of the hybrid protein causes it to remain in the membrane, protected from the rapid enzymatic degradation which would occur were the labeled protein to reside in the cytoplasm or in the lumen of the endocytic vesicle.

After insertion into the plane of the membrane of the endocytic vesicle, the labeled hybrid protein can "traf-

fic", as follows. The endocytic vesicle buds off from the cytoplasmic membrane and enters the cytoplasm of the cell, where it can merge with a lysosome into which the labeled hybrid protein is then incorporated. Alternatively, the endocytic vesicle can recycle to the cytoplasmic membrane of the cell. In either case, the label remains trapped in the target cell.

As is mentioned above, a major diagnostic use of the labeled hybrid proteins will be the *in vivo* and *in vitro* detection of metastatic loci, using conventional cell staining and labeling techniques. Such detection could be of particular value in surgery, by providing the surgeon with information needed to know how much tissue to excise when removing, e.g., metastatic melanoma cells.

Example 2. Construction of a cholera toxin A₁-diphtheria toxin B'-IL2 gene, and use of the resultant hybrid protein

Plasmid pCVD2 containing the coding sequence for the enzymatically-active A₁ fragment of cholera toxin (see Fig. 5) was prepared from a *Vibrio cholera* DNA library as described by Mekalanos et al. (Nature 306 :551-557, 1983). Fig. 6 outlines the strategy employed in engineering a cholera toxin A₁-diphtheria toxin B'-IL2 gene. Briefly, pCVD2 was cleaved with the restriction enzyme *Xba*I at the unique *Xba*I site. The following synthetic linker, which has 1/2 of an *Xba*I site at each end, was ligated to the linearized plasmid in order to introduce a *Nco*I site upstream from the *Xba*I site :

```

      5' C TAG ACC ATG GGA AAT GAT GAT AAG TTA-
      3'          TGG TAC CCT TTA CTA CTA TTC AAT-
peptide:          fmet-Gly-Asn-Asp-Asp-Lys-Leu-
                    1/2
                    XbaI

```

```

(cont.)      - TAT CGG GCA GAT T      - 3'
              - ATA GCC CGT CTA AGA TC - 5'
              - Tyr-Arg-Ala-Asp-Ser-Arg
                    1/2
                    XbaI

```

The appropriate construct was selected by restriction site mapping and sequence determinations, and then was digested with *Nco*I and *Cla*I to produce a *Nco*I-*Cla*I fragment. This in turn was digested with *Scr*FI. The 3' end of the resulting *Nco*I-*Scr*FI fragment was ligated to the following synthetic linker :

```

      5' - G GGT TCA GGG CC - 3'
      3' - CCA AGT C      - 5'
peptide: Pro-Gly-Ser-Gly-Pro
          1/2          1/2
          ScrFI       ApaI

```

The polypeptide encoded by the resulting *Nco*I-*Apa*I fragment lacks the natural cholera toxin signal sequence, having instead fmet-Gly followed by the mature A₁ region of cholera toxin, followed by Gly-Ser-Gly-Pro. This construct can be cloned into a plasmid that encodes diphtheria toxin fragment B' fused to the human interleukin-2 gene (plasmid pPA123, Fig. 7). Plasmid pPA123 was constructed from plasmid pDW124 (Diane Williams, Ph.D. dissertation, Boston University School of Medicine, Department of Microbiology, Boston, MA, 02118, 1989) as outlined in Fig. 7. Plasmid pDW124 encodes a diphtheria toxin fragment A-fragment B'-IL2 fusion protein that is expressed off the *trc* promoter in *E. coli*. The sequences encoding fragment A were deleted by digestion with the restriction endonucleases *Nco*I and *Nsi*I. The following oligonucleotides were used to rebuild the fragment A/B disulfide loop (I₁) sequence, introduce an *Apa*I site on the 5' end of the loop, and recreate the *Nco*I site encoding the translation-initiating ATG codon :

```

5' - C ATG GGG TCA GAT GGG CCC TGT GCA GGA AAT CGT GTC-
3' - CC AGT CTA CCC GGG ACA CGT CCT TTA GCA CAG-
5 peptide: fmet-Gly-Ser-Val-Gly-Pro-Cys-Ala-Gly-Asn-Arg-Val-
          1/2 ApaI
          NcoI

```

10 (cont.) -AGG CGA TCA GTA GGT AGC TCA TTG TCA TGC A - 3'
-TCC GCT AGT CAT CCA TCG AGT AAC AGT - 5'
-Arg-Arg-Ser-Val-Gly-Ser-Ser-Leu-Ser-Cys
 Sau3AI 1/2
 NsiI

15 Plasmid pPA123 resulted from ligating the above oligonucleotide fragment onto the NcoI-NsiI-digested pDW124 vector fragment. Plasmid pPA123 can now be used to fuse sequences encoding cholera toxin fragment A₁ to diphtheria toxin B'-IL2 as shown in Fig. 6. Plasmid pPA123 is digested with restriction enzymes NcoI and ApaI, and the resulting vector fragment is ligated to the modified cholera toxin fragment A₁ described above,
20 to yield a plasmid encoding a cholera toxin A₁-diphtheria toxin B'-IL2 hybrid ("CTA/DTB'/IL2 hybrid"), which is expressed from the *trc* promoter on the plasmid.

Following expression of the recombinant gene in E.coli, the CTA/DTB'/IL2 hybrid protein can be isolated and used in appropriate treatment regimens : for example, as an adjunct to treatment with diphtheria toxin-IL2 hybrid. Diphtheria toxin-IL2 hybrid effectively targets the cell-killing ability of diphtheria toxin to cells bearing the IL2 receptor, such as certain leukemic T-cells. However, the pharmacological effectiveness of diphtheria toxin-IL2 hybrid is diluted by circulating endogenous IL2, which is naturally synthesized by activated T-cells and which competes with diphtheria toxin-IL2 hybrid for IL2 receptors on T-cells. By first exposing the target cells to CTA/DTB'/IL2 hybrid, the biological activity of cholera toxin can be harnessed to alleviate this problem. The A₁ subunit of natural cholera toxin enzymatically catalyzes the ADP-ribosylation of a GTP-binding regulatory component of the adenylate cyclase complex, resulting in the accumulation of cyclic AMP within the affected cell and thereby disrupting a multitude of cellular functions without killing the cell. Targeting the cholera toxin A₁ activity specifically to cells bearing the IL2 receptor will result in the temporary inhibition of IL2 synthesis within those cells. This permits depletion of the amount of circulating IL2 available to compete with diphtheria toxin-IL2 for IL2 receptors, without interfering with expression of IL2 receptors on the surfaces of the T-cells and without injuring non-targeted cells. Subsequent treatment with diphtheria toxin-IL2 will thus be more effective at killing T-cells than if CTA/DTB'/IL2 hybrid had not been used.

Example 3. Construction of a Shiga-like toxin A-diphtheria toxin B'-IL2 gene, and use of the resultant hybrid protein

The DNA sequence and corresponding amino acid sequence for the A subunit of Shiga-like toxin ("SLT-A") are shown in Fig. 8. Bacteriophage H19B DNA from a strain of *E.coli* that produces SLT-A is prepared as described by Calderwood et al. (Proc. Natl. Acad. Sci. USA 84 :4364-4368, 1987) and digested with TaqI and XmnI. A TaqI-XmnI fragment (approx. 650 bp) corresponding to most of the coding sequence for SLT-A (the "sltA gene") is isolated therefrom (see Fig. 9) ; the following oligonucleotide is then ligated onto the 5'(TaqI) end of the fragment :

```

50      5' - CATG GGA AAG GAA TTT ACC TTA GAC TTC T   - 3'
        3' -      CCT TTC CTT AAA TGG AAT CTG AAG AGC - 5'
peptide: fmet-Gly-Lys-Glu-Phe-Thr-Leu-Asp-Phe-Ser
          1/2                               1/2
          NcoI                               TagI

```

55 This oligonucleotide sequence provides an fmet-Gly coding sequence followed by a sequence coding for the first eight amino acids of the mature SLT A subunit, to replace the section of the natural gene (coding for the toxin signal peptide and same eight amino acids of the mature SLT A subunit) which was cleaved off during TaqI digestion of the gene. Also provided by the oligonucleotide linker is a 1/2 NcoI site at the 5' end of the construct, to permit expression from the *trc* promoter of the hybrid plasmid.

The following oligonucleotide sequence, which regenerates the coding region (cleaved off by XmnI diges-

tion) for the carboxyl end of the SLT A subunit up to the initial Cys codon, and introduces an ApaI restriction site, is ligated to the 3' (XmnI) end of the sltA gene fragment :

5' - ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG-
 3' - TAA AGA AAA CCT TCG TAA TTA CGT TAA GAC-
 peptide: Ile-Ser-Phe-Gly-Ser-Ile-Asn-Ala-Ile-Leu-
 1/2
XmnI

(cont.) -GGA AGC GTG GCA TTA ATA CTG AAT GGG CC -3'
 -CCT TCG CAC CGT AAT TAT GAC TTA C -5'
 -Gly-Ser-Val-Ala-Leu-Ile-Leu-Asn-Gly-Pro
 1/2 ApaI

The NcoI-ApaI sltA gene sequence can be ligated into a NcoI + ApaI-digested plasmid pPA123 (Fig. 7) to yield a SLTA-diphtheria toxin B'-IL2 ("SLTA/DTB'/IL2 hybrid") gene that can be expressed in E.coli from the trc promoter on the plasmid (see Fig. 9).

An alternative cloning strategy for constructing a plasmid encoding a Shiga-like toxin A-diphtheria toxin B'-IL2 hybrid is illustrated in Fig. 10.

Purified SLTA/DTB'/IL2 hybrid protein would be useful as a treatment for conditions involving overproduction of cells bearing IL2 receptors, such as certain T-cell lymphomas and organ transplant rejection crises. As is the case for diphtheria toxin-IL2, the IL2 portion of the hybrid causes the hybrid to attach specifically to IL2-receptor-bearing cells, so that the enzymatic portion of the hybrid can be inserted into the targeted cell ; the enzymatic portions of both types of hybrid toxins then act on the protein synthesis machinery in the cell to shut down protein synthesis, thus killing the cell. The difference between these two types of hybrid toxins is the nature of their enzymatic activities : the enzymatic portion of diphtheria toxin-IL2 hybrid catalyzes the ADP-ribosylation by nicotinamide adenine dinucleotide of Elongation Factor 2, thereby inactivating this factor which is necessary for protein synthesis, while the enzymatic portion of SLTA/DTB'/IL2 hybrid is a ribonuclease capable of cleaving ribosomal RNA at a critical site, thereby inactivating the ribosome. SLTA/DTB'/IL2 hybrid would therefore be useful as a treatment for the same indications as diphtheria toxin-IL2 hybrid, and could be substituted if, for example, the proliferating T-cells develop a resistance to the latter hybrid toxin.

Example 4. Construction of ricin A-diphtheria toxin B'- IL2 gene, and use of the resultant hybrid protein

A genomic clone bank of castor bean (Ricinus communis) DNA is prepared as described in Halling et al., Nucl. Acids Res. 13 :8019-8033, 1985, and a ~780bp BanI fragment of the ricin gene, corresponding to most of the ricin A domain (the enzymatic domain) and a portion of the ricin A-to-B linker peptide, is isolated therefrom (see Fig. 11). The following synthetic oligonucleotide is ligated onto both ends of the fragment, phosphorylating only the bottom strand of DNA shown :

5' - C ATG GCT ATA TTC CCC AAA CAA TAC CCA ATT-
 3' - CGA TAT AAG GGG TTT GTT ATG GGT TAA-
 Peptide: fmet-Ala-Ile-Phe-Pro-Lys-Gln-Tyr-Pro-Ile-
 1/2
NcoI

(cont.) -ATA AAC TTT ACC ACA GCG G - 3'
 -TAT TTG AAA TGG TGT CGC CCA CG - 5'
 -Ile-Asn-Phe-Thr-Thr-Ala-Gly-Ala
 1/2
BanI

The resulting ligated fragment (illustrated in Fig. 11) is partially digested with Fspl, and the ~780bp band corresponding to a BanI-Fspl ricin A gene fragment with a NcoI-BanI linker at the 5' end is isolated (see Fig. 11). The NcoI-BanI linker supplies the mature ricin A N-terminal amino acid codons which were cleaved from the

fragment during BanI digestion, as well as the codons for fmet-Ala to replace the natural ricin A signal peptide.

The following oligonucleotide is ligated onto the 3' (FspI) blunt end of the fragment, phosphorylating only the top strand shown :

5' - GCA CCT CCA CCA TCG TCA CAG TTT GGG CC - 3'
 3' - CGT GGA GGT GGT AGC AGT GTC AAA C - 5'
 Peptide: Ala-Pro-Pro-Pro-Ser-Ser-Gln-Phe-Gly-Pro
 1/2 1/2
FspI ApaI

This linker supplies the ricin A coding sequence cleaved from the 3' end of the ricin A fragment during the FspI digest, plus a 1/2 ApaI site for fusion to plasmid pPA123.

The completed construct is then cloned into NcoI/ApaI-digested pPA123 to yield a ricin A-diphtheria toxin B'-IL2 gene that can be expressed in E.coli from the trc promoter on the plasmid (see Fig. 12).

Purified ricin A-diphtheria toxin B'-IL2 hybrid, like the SLTA/DTB'/IL2 hybrid of Example 3, inactivates ribosomes in cells bearing IL2 receptors, resulting in cessation of protein synthesis and death of the targeted cells. The ricin A hybrid would thus have the same applications as SLTA/DTB'/IL2 hybrid, as discussed in Example 3.

Example 5. Construction of phenylalanine hydroxylase- diphtheria toxin B gene, and use of the resultant hybrid protein.

A human liver cDNA library is screened for phenylalanine hydroxylase ("PH") cDNA as described by Kwok et al., Biochem. 24 :556-561, 1985. The approximately 1160-bp EcoRII-AflII fragment that encodes most of the PH protein is isolated (see Figs. 13 and 14). The following linkers are ligated onto the 5' EcoRII end in order to recreate the 5' coding sequences and incorporate an NcoI site :

5' - C ATG TCC ACT GCG GTC CTG GAA AAC - 3'
 3' - AGG TGA CGC CAG GAC CTT TTG GGT CC - 5'
 fmet-Ser-Thr-Ala-Val-Leu-Gln-Asn-Pro-Gly
NcoI 1/2
 sticky end EcoRII

The following linkers are ligated onto the 3' AflII end to complete the PH coding sequence and to include an ApaI restriction site in the correct translational reading frame for fusion to diphtheria toxin fragment B sequences (Fig. 14) :

5' - TT AAG ATT TTG GCT GAT TCC ATT AAC AGT GAA ATT GGA-
 3' - C TAA AAC CGA CTA AGG TAA TTG TCA CTT TAA CCT-
 Lys-Ile-Leu-Ala-Asp-Ser-Ile-Asn-Ser-Glu-Ile-Gly-
 1/2
AflII

(cont.) -ATC CTT TGC AGT GCC CTC CAG AAA ATA AAG GGG CC - 3'
 -TAG GAA ACG TCA CGG GAG GTC TTT TAT TTC C - 5'
 -Ile-Leu-Cys-Ser-Ala-Leu-Gln-Lys-Ile-Lys-Gly-Pro
 1/2
ApaI

This fragment is then ligated onto the NcoI-ApaI digested pPA123 vector (Fig. 14) resulting in a plasmid that encodes phenylalanine hydroxylase fused to diphtheria toxin B'-IL-2. Finally, this plasmid is digested with EcoRI and SphI to remove IL-2 encoding sequences, which are replaced by the approximately 230 bp SphI-EcoRI fragment of corynebacteriophage β that encodes the 3' end of diphtheria toxin fragment B (Fig. 14). This completed construct codes for a PH-diphtheria toxin B hybrid protein that can be expressed in E.coli from the trc promoter on the plasmid (see Fig. 14).

The inherited disorder phenylketonuria, in which the inability to metabolize phenylalanine leads to an

accumulation of excess phenylalanine and possible brain damage in affected individuals, has been attributed to a genetic deficiency of the enzyme PH. By constructing a molecule in which active PH enzyme is linked to the cell-binding and translocation domains of diphtheria toxin Fragment B, the enzyme can be targeted to and incorporated into the broad range of cells which native diphtheria toxin normally attacks, achieving the widespread therapy that is called for by a defect such as phenylketonuria. This cloning strategy would be applicable to the construction of other hybrids useful in the treatment of other genetic defects.

Example 6. Construction of an HIV protease-binding protein-diphtheria toxin B'-IL2 gene, and use of the resultant hybrid protein.

A recombinant gene expressing a novel protein, an antigen-binding, single-polypeptide-chain analog of a monoclonal antibody composed of an antibody variable light-chain amino acid sequence (V_L) linked to a variable heavy-chain sequence (V_H) by a linker peptide, is constructed by the method of Bird et al., Science 242 :423-426, 1988, based upon the V_L and V_H sequences of a monoclonal antibody specific for and able to inactivate HIV protease (Hansen et al., Embo J. 7 :1785-1791, 1988) and a linker peptide designed by the method of Bird et al. The ends of the V_L -linker- V_H gene are modified with appropriate restriction enzymes and synthetic DNA linkers in order to produce an intact V_L -linker- V_H gene having 1/2 of a *Nco*I site at the 5' end and 1/2 of an *Apal* site at the 3' end. The gene is then cloned into *Nco*I + *Apal*-digested pPA123 to produce a plasmid expressing, from the *trc* promoter, an HIV protease-binding protein-diphtheria toxin B'-IL2 hybrid protein ("HIVP-BP/DTB'/IL2 hybrid").

Following expression of the recombinant gene in *E. coli*, the HIVP-BP/DTB'/IL2 hybrid protein can be isolated and used to treat an HIV infection in a human patient. The HIV virus infects and proliferates within T-cells, commandeering the cellular protein synthesis machinery to produce multiple copies of its own proteins. One viral protein in particular, the HIV protease, plays a critical role in the processing of other viral proteins; identifying a way to inactivate this protease within the infected cell has been the focus of much recent effort toward developing an effective AIDS therapy (see, e.g., Hansen et al.). The HIVP-BP/DTB'/IL2 hybrid delivers a viral protease-specific inhibitor specifically to activated T-cells bearing the IL2 receptor, and thus can be effective at low dosages, with little or no toxicity to other types of cells. This technology could be applied as well to other viral infections or genetic disorders.

Other Embodiments

Other embodiments are within the following claims. For example, any cell-specific polypeptide ligand can be used which has a binding domain specific for the particular class of cells which are to be labeled. Polypeptide hormones are useful such ligands. Hybrid protein made using the binding domain of α or β MSH, for example, can selectively bind to melanocytes, rendering hybrids, once labelled with a detectable label, useful in the diagnosis of melanoma and the *in vivo* and *in vitro* detection of metastatic melanoma loci. Such a hybrid, when attached to an enzymatically-active portion of a toxin molecule instead of to a detectable label, could be utilized to deliver that toxic activity specifically to the target melanoma cells. Other ligands provide different specificities: e.g., the binding domain of substance P recognizes receptors on the surfaces of neurons involved in the transmission of pain, so that labeled hybrids made using substance P can be used to map areas of the nervous system containing substance P receptors. Other specific-binding ligands which can be used include insulin, somatostatin, EGF, and Interleukins I, II, III, IV and VI. Interleukin II is of particular importance because of its role in allergic reactions and autoimmune diseases such as Systemic Lupus Erythmatosis (SLE), involving activated T cells. Other useful polypeptide ligands having cell-specific binding domains are follicle stimulating hormone (specific for ovarian cells), luteinizing hormone (specific for ovarian cells), thyroid stimulating hormone (specific for thyroid cells), vasopressin (specific for uterine cells, as well as bladder and intestinal cells), prolactin (specific for breast cells), and growth hormone (specific for certain bone cells). Alternatively, a relatively indiscriminate cell-binding ligand (such as that of diphtheria toxin or ricin toxin) capable of binding to a wide variety of cell types in an organism can be used to effect widespread introduction of a specific chemical entity into cells of that organism, where more specific targeting is not the goal.

For a number of cell-specific ligands, the region within each such ligand in which the binding domain is located is now known. Furthermore, recent advances in solid phase polypeptide synthesis enable those skilled in this technology to determine the binding domain of practically any such ligand, by synthesizing various fragments of the ligand and testing them for the ability to bind to the class of cells to be labeled. Thus, the hybrid molecules of the invention need not include an entire ligand, but rather may include only a fragment of a ligand which exhibits the desired cell-binding capacity. Likewise, analogs of the ligand or its cell-binding region having minor sequence variations may be synthesized, tested for their ability to bind to cells, and incorporated into

the hybrid molecules of the invention. Other potential ligands include monoclonal antibodies or antigen-binding, single-chain analogs of monoclonal antibodies, where the antigen is a receptor or other moiety expressed on the surface of the target cell membrane.

The translocation function of the hybrid molecule may be supplied by an appropriate piece of a polypeptide other than diphtheria toxin, but which is capable of translocating in a manner analogous to that of diphtheria toxin (e.g., *Pseudomonas* exotoxin A, botulinum, neurotoxin, or ricin), or in any other manner which accomplishes the objective of translocating the functional "third part" of the hybrid molecule into the cell's cytoplasm.

The chemical entity to be inserted into the cell can vary widely and still be within the invention. For example, the enzyme which is genetically deficient in Tay-Sachs disease, hexosaminidase A, could be attached to a hybrid having a cell-binding domain specific for the cells most affected by the disease, nerve cells. Patients suffering from type 2 glycogenosis might be treated with a hybrid comprising α -1,4-glucosidase linked to the translocation segment of diphtheria toxin linked to insulin, which would largely target muscle cells, hepatocytes, and lymphocytes. (See Poznansky et al., Science 223 :1304-1306, 1984.) These are simply examples : any other enzyme deficiency disease for which the natural enzyme or its gene has been sequenced (or is amenable to sequencing by one skilled in the art, without undue experimentation) could be treated with a hybrid comprising the active enzyme linked to a translocation domain linked to an appropriate cell-binding ligand.

Intracellular viral and bacterial infections could be treated by an appropriate hybrid : for example, a hybrid which delivers into the cell a potent antibiotic, or a recombinant V_L -linker- V_H antigen-binding polypeptide which specifically binds viral particles or proteins.

Likewise, the hybrid of the invention will be useful for specifically destroying certain cells. Besides the cholera toxin A_1 -hybrid, ricin A-hybrid and Shiga-like toxin A-hybrid exemplified above, a cell-killing function may be provided by the enzymatically-active portion of any polypeptide toxin, including but not limited to LT toxin, C3 toxin, Shiga toxin, pertussis toxin, tetanus toxin and *Pseudomonas* exotoxin A. Cells to be targeted might include cancer cells, virus-infected cells, or adipocytes.

The invention includes biologically active mutational analogs of hybrid polypeptides described above. By manipulating the recombinant DNA sequence encoding the subject hybrid polypeptide using methods well known to those of ordinary skill in the art of genetic engineering, a series of mutations involving deletions and/or substitutions of individual or multiple base pairs in such recombinant DNA sequence is first created. Each such mutated sequence is then inserted into an expression vector and expressed in an appropriate expression system. The biological activity of the mutational analog so produced can then be compared to that exhibited by the hybrid molecule of which it is an analog (the "parent polypeptide"). The particular assay used will depend upon the particular enzymatic activity and cell-binding specificity of the parent polypeptide. For example, mutational analogs of the Shiga-like toxin A/diphtheria toxin B'/IL2 (SLTA/DTB'/IL2) hybrid, the cholera toxin A_1 /diphtheria toxin B'/IL2 (CTA/DTB'/IL2) hybrid, and the ricin A/diphtheria toxin B'/IL2 hybrid may be tested and compared to their respective parent polypeptides in the following cell cytotoxicity assay, which is specific for toxins capable of binding to IL2 receptor-bearing cells.

Assay

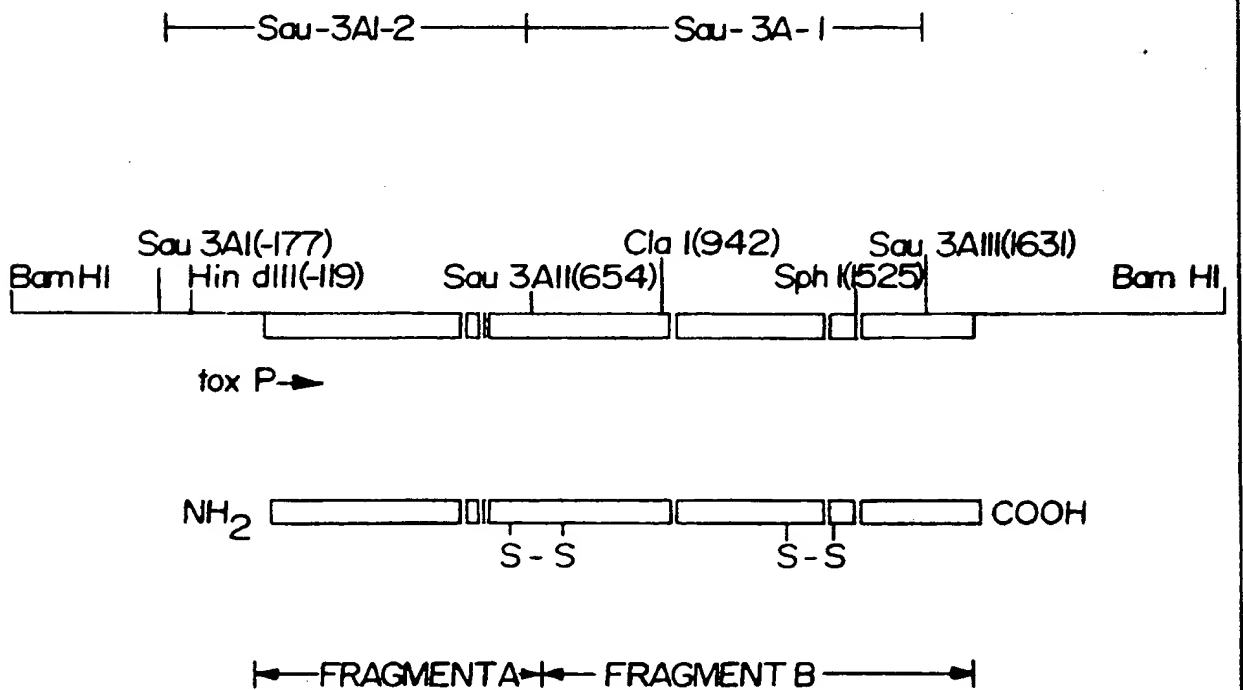
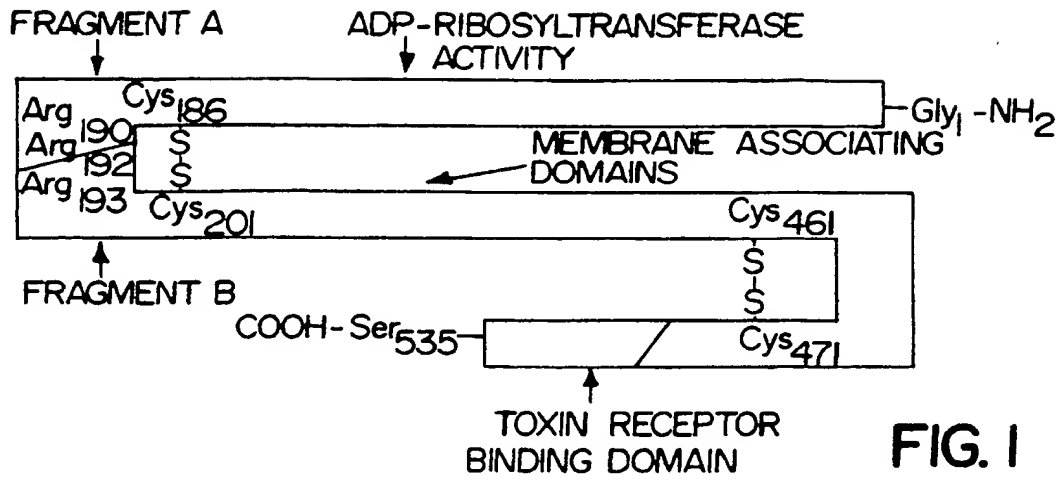
Cultured HUT 102/6TG (Tsuda et al., Proc. Natl. Acad. Sci. USA 83 :9694, 1986) or YT2C2 (Teshigawari et al., J. Exp. Med. 165 :223, 1987) cells are maintained in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Collect, GIBCO), 2mM glutamine, and penicillin and streptomycin to 50 IU and 50 μ g/ml, respectively. Cells are seeded in 96-well V-bottomed plates (Linbro-Flow Laboratories, McLean, VA) at a concentration of 5×10^4 per well in complete medium. Putative toxins are added to varying concentrations (10^{-12} M to 10^{-6} M) and the cultures are incubated for 18 hrs. at 37°C in a 5% CO₂ atmosphere. Following incubation, the plates are centrifuged for 5 min. at 170 x g, and the medium removed and replaced with 200 μ l leucine-free medium (MEM, Gibco) containing 1.0 μ Ci/ml [¹⁴C]-leucine (New England Nuclear, Boston, MA). After an additional 90 min. at 37°C, the plates are centrifuged for 5 min. at 170 x g, the medium is removed and the cells are lysed by the addition of 4 M KOH. Protein is precipitated by the addition of 10% trichloroacetic acid and the insoluble material is then collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters are washed, dried, and counted according to standard methods. Cells cultured with medium alone serve as the control.

Where IL4 replaces IL2 as the cell-binding portion of the resulting hybrid, the hybrid and its mutational analogs may be tested by a similar assay utilizing CT4R cells (William E. Paul, NIH), P815 cells (ATCC), or CTLL2 (ATCC), seeded at 1×10^4 cells per well and incubated for 40 hours.

Claims

- 5 1. A hybrid molecule comprising a first part, a second part, and a third part connected by covalent bonds,
 (a) said first part comprising a portion of the binding domain of a cell-binding ligand, said portion being effective to cause said hybrid molecule to bind to a cell of an animal ;
 (b) said second part comprising a portion of a translocation domain of a protein capable of translocating
 10 said third part across the cytoplasmic membrane of said cell ; and
 (c) said third part comprising a chemical entity to be introduced into said cell, provided that (i) said hybrid molecule is produced by expression of a recombinant DNA molecule encoding said hybrid molecule, and (ii) said second part and said third part are not segments of the same naturally-occurring polypeptide toxin.
- 15 2. A hybrid molecule comprising a first part, a second part, and a third part connected by covalent bonds,
 (a) said first part comprising a portion of the binding domain of a cell-binding ligand, said portion being effective to cause said hybrid molecule to bind to a cell of an animal ;
 (b) said second part comprising a portion of a translocation domain of a protein capable of translocating
 20 said third part across the cytoplasmic membrane of said cell ; and
 (c) said third part comprising a chemical entity to be introduced into said cell, provided that (i) said first part and said second part are not segments of the same naturally-occurring polypeptide toxin, and (ii) said second part and said third part are not segments of the same naturally-occurring polypeptide toxin.
- 25 3. The hybrid molecule of claim 1 or claim 2, wherein said protein is a naturally-occurring toxin.
4. The hybrid molecule of claim 2, wherein said protein is selected from the group consisting of diphtheria toxin and Pseudomonas exotoxin A.
- 30 5. The hybrid molecule of claim 1 or claim 2, wherein said ligand is a polypeptide hormone.
6. The hybrid molecule of claim 5, wherein said hormone is selected from the group consisting of insulin, Interleukin II, Interleukin IV, Interleukin VI, and EGF.
- 35 7. The hybrid molecule of claim 2, wherein said ligand is a steroid hormone.
8. The hybrid molecule of claim 1 or claim 2, wherein said ligand is an antigen-binding, single-chain analog of a monoclonal antibody, and said antigen is expressed on the surface of said cell.
- 40 9. The hybrid molecule of claim 1 or claim 2, wherein said ligand is a polypeptide toxin capable of binding to said cell.
10. The hybrid molecule of claim 2, wherein any adjacent two of said parts are both polypeptides, and said covalent bond between said two parts is a peptide bond.
- 45 11. The hybrid molecule of claim 10, wherein said first part and said third part each comprises a polypeptide and said hybrid molecule comprises a recombinant protein.
12. The hybrid molecule of claim 1 or claim 2, wherein said chemical entity comprises an enzymatically-active portion of an enzyme.
- 50 13. The hybrid molecule of claim 12, wherein said enzyme is a toxin.
14. The hybrid molecule of claim 13, wherein said enzyme is selected from the group consisting of cholera toxin, LT toxin, C3 toxin, Shiga toxin, E.coli Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin,
 55 diphtheria toxin and Pseudomonas exotoxin A.
15. The hybrid molecule of claim 14, wherein said enzyme is cholera toxin.
16. The hybrid molecule of claim 12, wherein said enzyme is selected from the group consisting of hexosaminidase A, α -1,4-glucosidase, phenylalanine hydroxylase, a protease and a nuclease.

17. The hybrid molecule of claim 12, wherein said enzymatically-active portion supplies an enzymatic activity in which said cell is deficient.
18. The hybrid molecule of claim 17, wherein said deficiency is genetic.
19. The hybrid molecule of claim 1 or claim 2, wherein said chemical entity comprises an antigen-binding, single-chain analog of a monoclonal antibody.
20. The hybrid molecule of claim 19, wherein said antigen is a viral protein.
21. The hybrid molecule of claim 20, wherein said viral protein is HIV protease.
22. A hybrid polypeptide molecule comprising CTA/DTB'/IL2 hybrid.
23. A hybrid polypeptide molecule comprising SLTA/DTB'/IL2 hybrid.
24. A hybrid polypeptide molecule comprising ricin A-diphtheria toxin B'-IL2 hybrid.
25. A hybrid polypeptide molecule encoded by the phenylalanine hydroxylase-diphtheria toxin fragment B gene of Fig. 14.
26. A hybrid polypeptide molecule comprising HIVP-BP/DTB'/IL2 hybrid.
27. A hybrid polypeptide molecule comprising a biologically active mutational analog of the hybrid molecule of any of claims 22-26.
28. A hybrid polypeptide molecule comprising an enzymatically-active portion of Shiga-like toxin A and a portion of a binding domain of a cell-binding polypeptide ligand, said portion of said binding domain being effective to cause said hybrid molecule to bind to a cell of an animal.
29. The hybrid molecule of claim 28, wherein said polypeptide ligand is IL2.
30. A method of treating an animal having a disease characterized by the enzyme deficiency of claim 17, said method comprising administering to said animal an effective amount of the hybrid molecule of claim 17.
31. A method of raising the cyclic AMP level within a target cell, said method comprising contacting said target cell with the hybrid molecule of claim 15.
32. The method of claim 31, wherein said target cell is a T cell and said hybrid molecule comprises at least a portion of the binding domain of Interleukin II.
33. A method of treating a human patient infected with HIV, said method comprising administering to said patient an effective amount of the hybrid molecule of claim 21.
34. A recombinant DNA molecule encoding the hybrid protein of claim 1 or claim 2.
35. A vector comprising the recombinant DNA molecule of claim 34.
36. A cell containing the recombinant DNA molecule of claim 34.
37. The cell of claim 36, wherein said cell is capable of expressing said recombinant DNA molecule.
38. A method of preparing the hybrid molecule of claim 1 or claim 2, said method comprising providing a cell containing a recombinant DNA molecule encoding said hybrid molecule (the "transformed cell"), and permitting said transformed cell to express said recombinant DNA molecule.



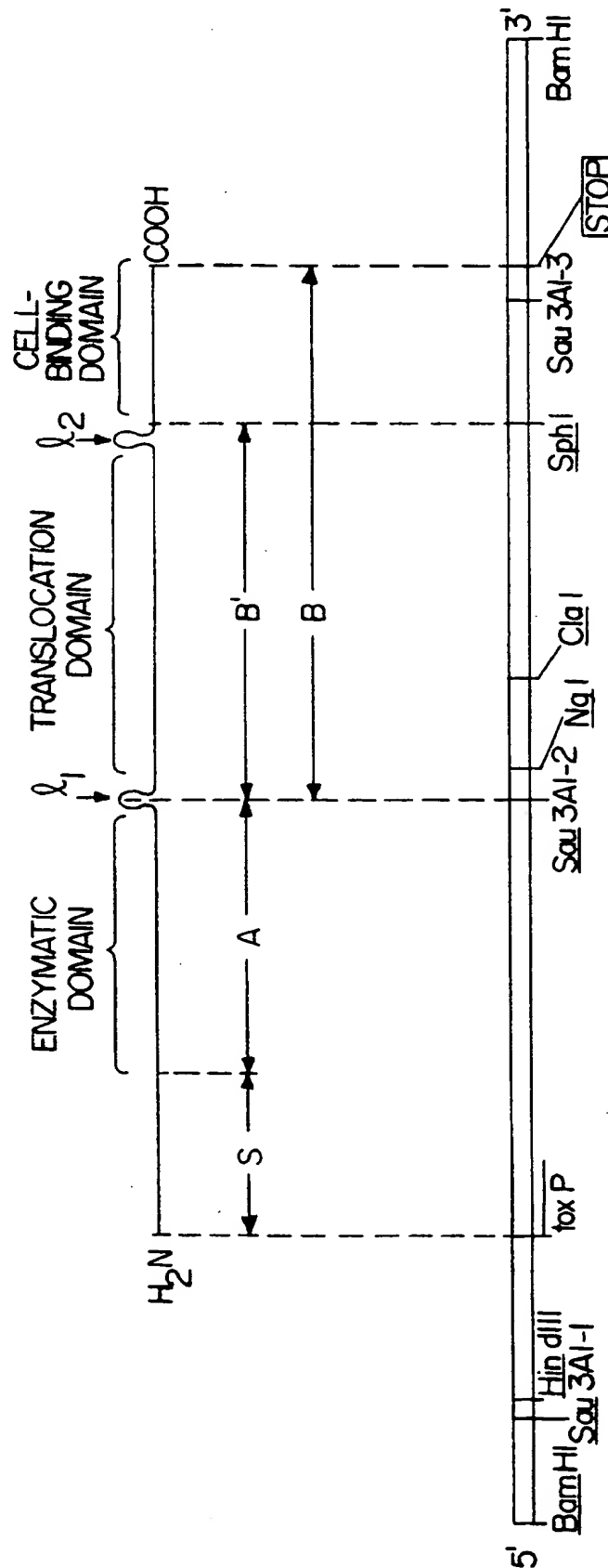


FIG. 3

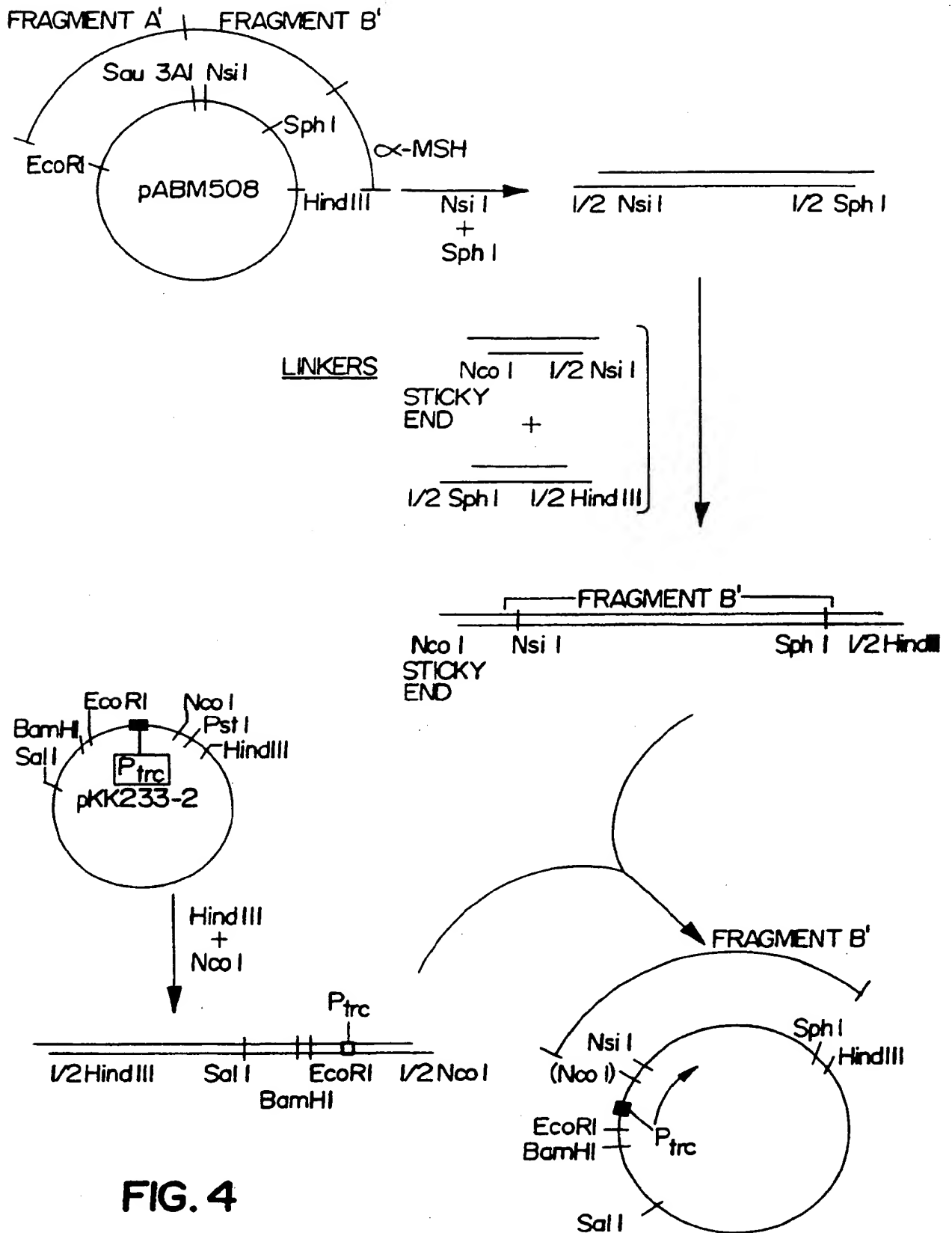


FIG. 4

FIG. 5-1

10	20	30	40	50	60
ATGGTAAAGATAATATTTGTGTTTTTATTTTCTTATCATCATTTTTCATATGCAAATGAT					
<u>MET</u> VaI Lys I I e I I e Phe Va I Phe Phe I I e Phe Leu Ser Ser Phe Ser Tyr Al a Asn Asp					
70	80	90	100	110	120
GATAAGTTATATCGGGCAGATTCTAGACCTCCTGATGAAATAAAGCAGTCAGGTGGTCTT					
Asp Lys Leu Tyr Arg Ala Asp Ser Arg Pro Pro Asp Glu I I e Lys Gl n Ser Gly Gly Leu					
130	140	150	160	170	180
ATGCCAAGAGGACAGAGTCAGTACTTTGACCGAGGTACTCAAATGAATATCAACCTTTAT					
<u>MET</u> Pro Arg Gly Gl n Ser Gl n Tyr Phe Asp Arg Gly Thr Gl n <u>MET</u> Asn I I e Asn Leu Tyr					
190	200	210	220	230	240
GATCATGCAAGAGGAACTCAGACGGGATTTGTTAGGCACGATGATGGATATGTTTCCACC					
Asp His Ala Arg Gly Thr Gl n Thr Gly Phe Va I Arg His Asp Asp Gly Tyr Va I Ser Thr					
250	260	270	280	290	300
TCAATTAGTTTGAGAAGTGCCCACTTAGTGGGTCAAACCTATATTGTCTGGTCATTCTACT					
Ser I I e Ser Leu Arg Ser Ala His Leu Va I Gly Gl n Thr I I e Leu Ser Gly His Ser Thr					
310	320	330	340	350	360
TATTATATATATGTTATAGCCACTGCACCCAACATGTTTAACGTTAATGATGTATTAGGG					
Tyr Tyr I I e Tyr Va I I I e Ala Thr Ala Pro Asn <u>MET</u> Phe Asn Va I Asn Asp Va I Leu Ala					
370	380	390	400	410	420
GCATACAGTCCTCATCCAGATGAACAAGAAGTTTCTGCTTTAGGTGGGATTCCATACTCC					
Al a Tyr Ser Pro His Pro Asp Glu Gl n Glu Va I Ser Ala Leu Gly Gly I I e Pro Tyr Ser					

FIG. 5-2

430 440 450 460 470 480
 CAAATATATGGATGGTATCGAGTTCATTTTGGGGTGCTTGATGAACAATTACATCGTAAT
 GlnIleTyrGlyTrpTyrArgValHisPheGlyValLeuAspGluGlnLeuHisArgAsn
 490 500 510 520 530 540
 AGGGGCTACAGAGATAGATATTACAGTAACTTAGATATTGCTCCAGCAGCAGATGGTTAT
 ArgGlyTyrArgAspArgTyrTyrSerAsnLeuAspIleAlaProAlaAlaAspGlyTyr
 550 560 570 580 590 600
 GGATTGGCAGGTTTCCCTCCGGAGCATAGAGCTTGGAGGGAAGAGCCGTGGATTTCATCAT
 GlyLeuAlaGlyPheProProGluHisArgAlaTrpArgGluGluProTrpIleHisHis
 Scr Fl
 610 620 630 640 650 660
 GCACCGCCGGGTTGTGGGAATGCTCCAAGATCATCGATGAGTAATACTTGCGATGAAAAA
 AlaProProGlyCysAlaAsnAlaProArgSerSerMETSerAsnThrCysAspGluLys
 670 680 690 700 710 720
 ACCCAAAGTCTAGGTGTAAAATTCCTTGACGAATACCAATCTAAAGTTAAAAGACAAATA
 ThrGlnSerLeuGlyValLysPheLeuAspGluTyrGlnSerLysValLysArgGlnIle
 730 740 750 760 770 STOP 780
 TTTTCAGGCTATCAATCTGATATTGATACACATAATAGAATTAAGGATGAATTATGATTA
 PheSerGlyTyrGlnSerAspIleAspThrHisAsnArgIleLysAspGluLeu---Leu

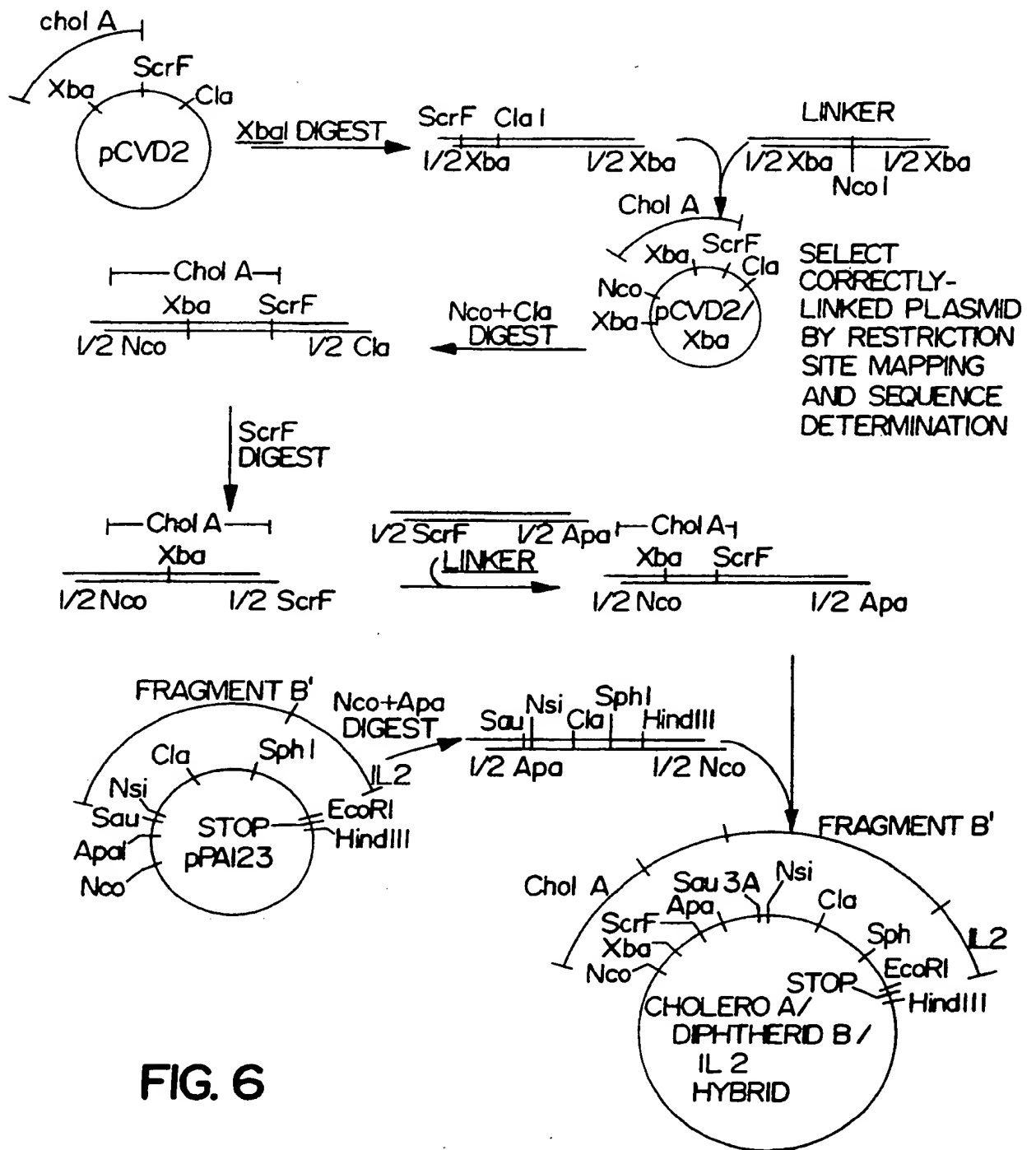


FIG. 7

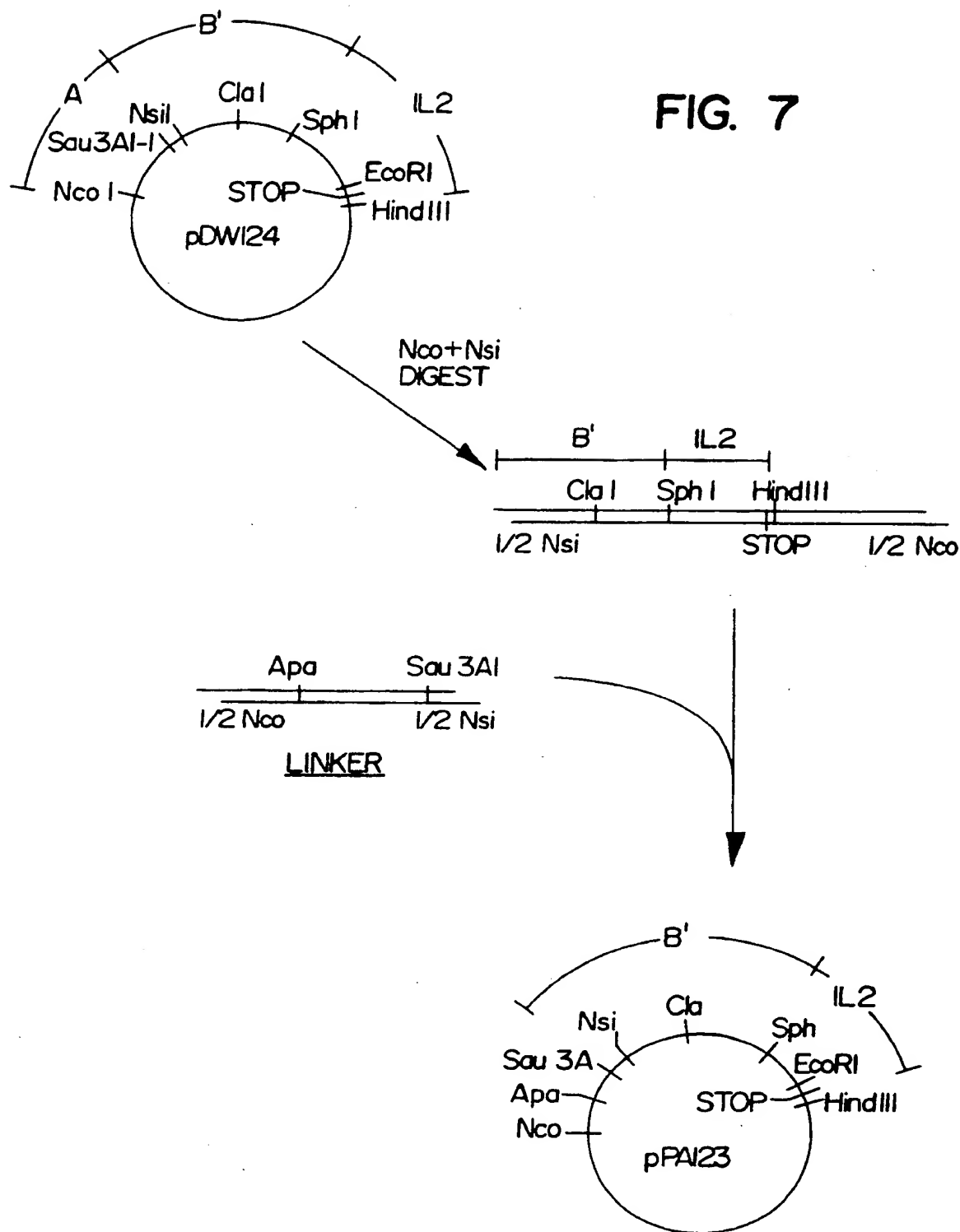


FIG. 8-1

10 20 30 40 50 60
 | | | | |
 ATGAAAATAATTATTTTGTAGAGTGCTAACTTTTTTCTTTGTTATCTTTTCAGTTAATGTG
METLysIleIleIlePheArgValLeuThrPhePhePheValIlePheSerValAsnVal
 70 80 90 100 110 120
 | | | | |
 GTGGCGAAGGAATTTACCTTAGACTTCTCGACTGCAAAGACGTATGTAGATTCGCTGAAT
 ValAlaLysGluPheThrLeuAspPheSerThrAlaLysThrTyrValAspSerLeuAsn
 130 140 150 160 170 180
 | | | | |
 GTCATTCGCTCTGCAATAGGTACTCCATTACAGACTATTTTCATCAGGAGGTACGTCTTTA
 ValIleArgSerAlaIleGlyThrProLeuGlnThrIleSerSerGlyGlyThrSerLeu
 190 200 210 220 230 240
 | | | | |
 CTGATGATTGATAGTGGCTCAGGGGATAATTTGTTTGCAGTTGATGTCAGAGGGATAGAT
 LeuMETIleAspSerGlySerGlyAspAsnLeuPheAlaValAspValArgGlyIleAsp
 250 260 270 280 290 300
 | | | | |
 CCAGAGGAAGGGCGGTTTAATAATCTACGGCTTATTGTTGAACGAAATAATTTATATGTG
 ProGluGluGlyArgPheAsnAsnLeuArgLeuIleValGluArgAsnAsnLeuTyrVal
 310 320 330 340 350 360
 | | | | |
 ACAGGATTTGTTAACAGGACAAATAATGTTTTTATCGCTTTGCTGATTTTTCACATGTT
 ThrGlyPheValAsnArgThrAsnAsnValPheTyrArgPheAlaAspPheSerHisVal
 370 380 390 400 410 420
 | | | | |
 ACCTTTCCAGGTACAACAGCGGTTACATTGTCTGGTGACAGTAGCTATACCACGTTACAG
 ThrPheProGlyThrThrAlaValThrLeuSerGlyAspSerSerTyrThrThrLeuGln

FIG. 8-2

430 440 450 460 470 480
 CGTGTTCAGGGATCAGTCGTACGGGGATGCAGATAAATCGCCATTCGTTGACTACTTCT
 ArgValAlaGlyIleSerArgThrGlyMETGlnIleAsnArgHisSerLeuThrThrSer
 490 500 510 520 530 540
 TATCTGGATTTAATGTCGCATAGTGGAACCTCACTGACGCAGTCTGTGGCAAGAGCGATG
 TyrLeuAspLeuMETSerHisSerGlyThrSerLeuThrGlnSerValAlaArgAlaMET
 550 560 570 580 590 600
 TTACGGTTTGTACTGTGACAGCTGAAGCTTTACGTTTTTCGGCAAATACAGAGGGGATTT
 LeuArgPheValThrValThrAlaGluAlaLeuArgPheArgGlnIleGlnArgGlyPhe
 610 620 630 640 650 660
 CGTACAACACTGGATGATCTCAGTGGGCGTTCTTATGTAATGACTGCTGAAGATGTTGAT
 ArgThrThrLeuAspAspLeuSerGlyArgSerTyrValMETThrAlaGluAspValAsp
 670 680 690 700 710 720
 CTTACATTGAACTGGGGAAGGTTGAGTAGCGTCCTGCCTGACTATCATGGACAAGACTCT
 LeuThrLeuAsnTrpGlyArgLeuSerSerValLeuProAspTyrHisGlyGlnAspSer
 730 740 750 760 770 780
 GTTCGTGTAGGAAGAATTTCTTTTGAAGCATTAAATGCAATTCTGGGAAGCGTGGCATT
 ValArgValGlyArgIleSerPheGlySerIleAsnAlaIleLeuGlySerValAlaLeu
 790 800 810 820 830 840
 ATACTGAATTGTCATCATCATGCATCGCGAGTTGCCAGAATGGCATCTGATGAGTTTCCT
 IleLeuAsnCysHisHisHisAlaSerArgValAlaArgMETAlaSerAspGluPhePro

Xmn I
 730 740 750 760 770 780
 GTTCGTGTAGGAAGAATTTCTTTTGAAGCATTAAATGCAATTCTGGGAAGCGTGGCATT
 ValArgValGlyArgIleSerPheGlySerIleAsnAlaIleLeuGlySerValAlaLeu

Nsi
 790 800 810 820 830 840
 ATACTGAATTGTCATCATCATGCATCGCGAGTTGCCAGAATGGCATCTGATGAGTTTCCT
 IleLeuAsnCysHisHisHisAlaSerArgValAlaArgMETAlaSerAspGluPhePro

FIG. 8-3

850 860 870 880 890 900
 TCTATGTGTCCGGCAGATGGAAGAGTCCGTGGGATTACGCACAATAAAATATTGTTGTGG
 SerMETCysProAlaAspGlyArgValArgGlyIleThrHisAsnLysIleLeuLeuTrp
 910 920 930 940 950 960
 GATTCATCCACTCTGGGGGCAATTCTGATGCGCAGAACTATTAGCAGTTGAGGGGGTAAA
 AspSerSerThrLeuGlyAlaIleLeuMETArgArgThrIleSerSer---GlyGlyLys
 970 980 990 1000 1010 1020
 ATGAAAAAACATTATTAATAGCTGCATCGCTTTCATTTTTTTCAGCAAGTGCGCTGGCG
METLysLysThrLeuLeuIleAlaAlaSerLeuSerPhePheSerAlaSerAlaLeuAla
 1030 1040 1050 1060 1070 1080
 ACGCCTGATTGTGTAAGTGGAAAGGTGGAGTATACAAAATATAATGATGACGATACCTTT
 ThrProAspCysValThrGlyLysValGluTyrThrLysTyrAsnAspAspThrPhe
 1090 1100 1110 1120 1130 1140
 ACAGTTAAAGTGGGTGATAAAGAATTATTTACCAACAGATGGAATCTTCAGTCTCTTCTT
 ThrValLysValGlyAspLysGluLeuPheThrAsnArgTrpAsnLeuGlnSerLeuLeu
 1150 1160 1170 1180 1190 1200
 CTCAGTGCGCAAATTACGGGGATGACTGTAACCATTAATACTAATGCCTGTCATAATGGA
 LeuSerAlaGlnIleThrGlyMETThrValThrIleLysThrAsnAlaCysHisAsnGly
 1210 1220 1230
 GGGGGATTTCAGCGAAGTTATTTTTCGTTGA
 GlyGlyPheSerGluValIlePheArg---

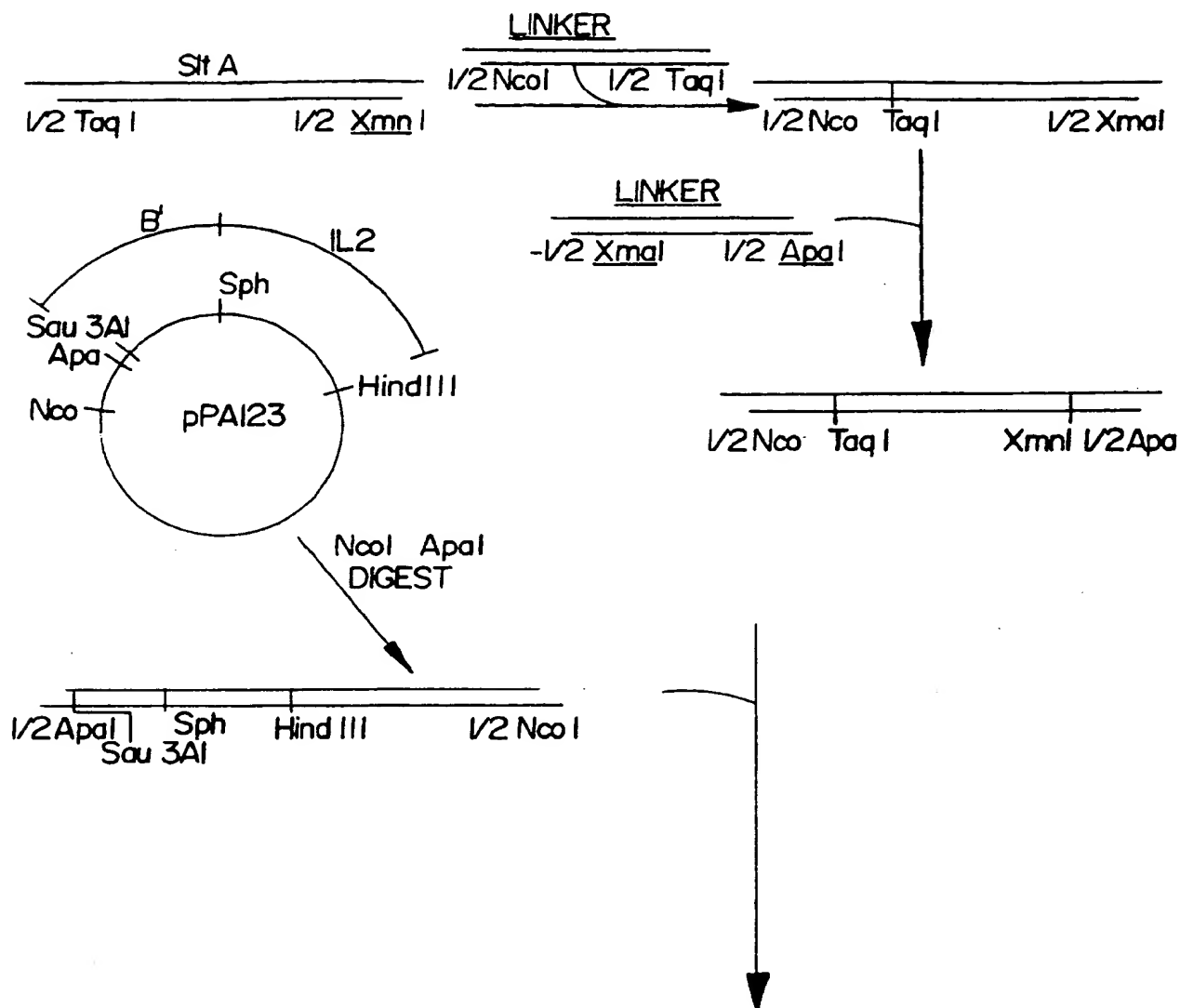
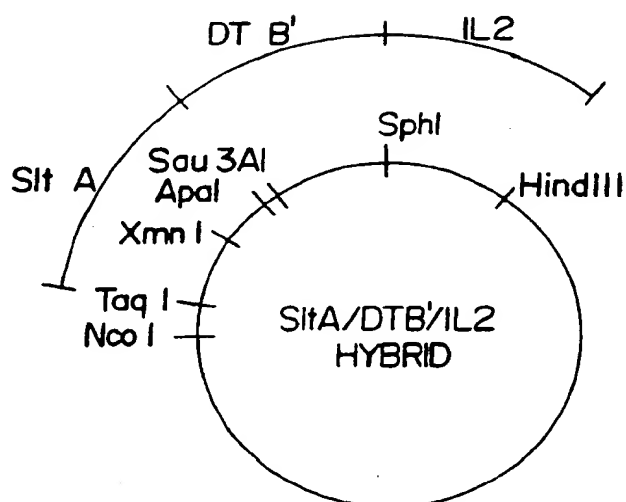
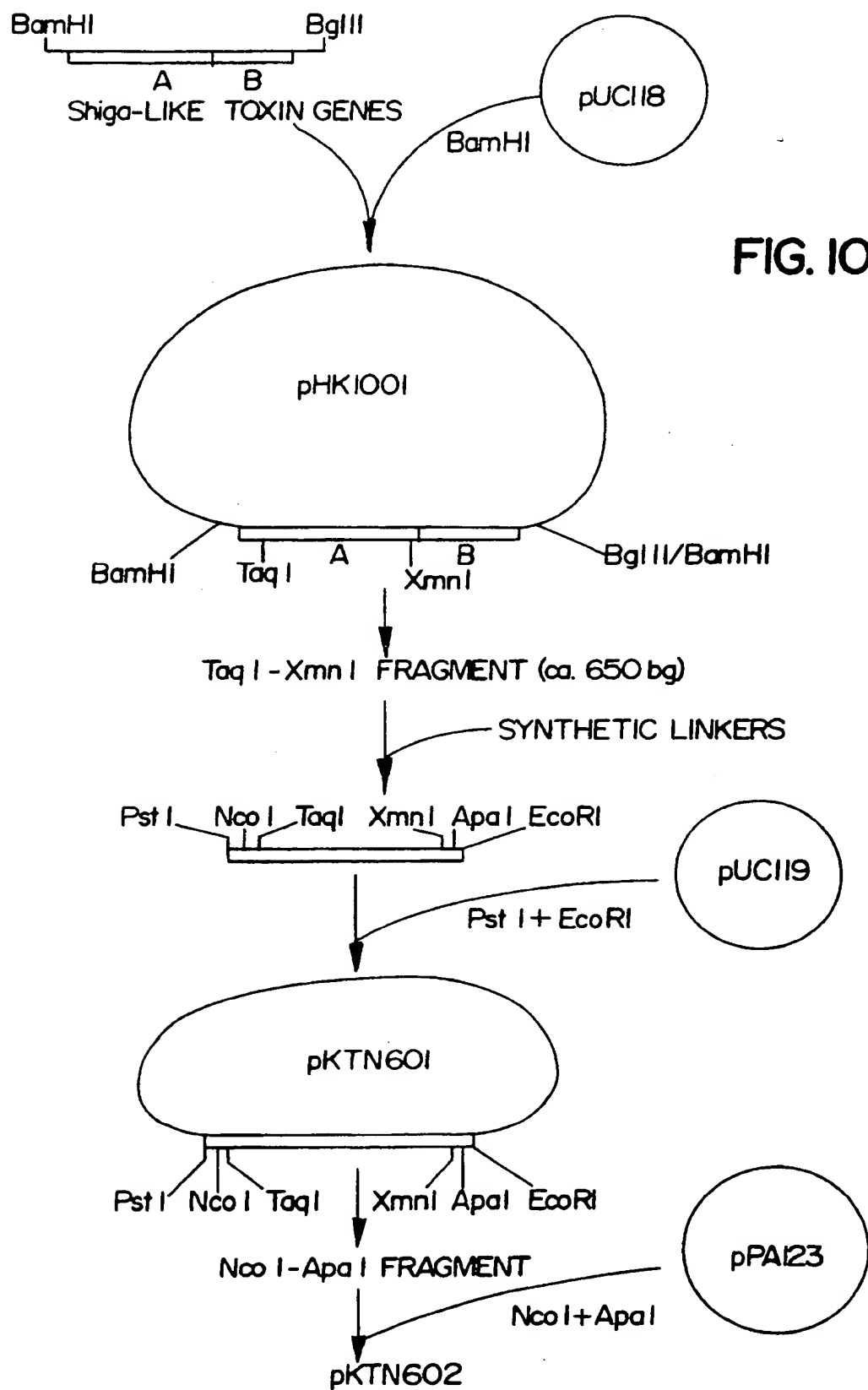


FIG. 9





1
 TCGACATTATATGATTTTAAATCAATTCCGTTTCTAATTTATAATTATTTTCGTTAAACCAATCAA
 66
 TTCCCTTTAAACACTGCTTATGCATATTCTGTCTCAAITTTATATATGGCATTGCATTCTTCCGTAT
 132
 TAATTTATAAGTTCACTTTTTATTGATCAAGTATTTGTGGTTTTCTTTATATAAAAAAATGTATTA
 198
 GTGTTTTTCTGTATTAATTTTATAAGTTCATCTTTATGAGAATGCTAATGTATTTGGACAGCCAAT
 264
 AAAATTCCAGAATTGCTGCAATCAAGGATGAAACCGGGAGGAAATACTATTGTAATATGGATGTAT
 330
 A V A T W L C F G S T S G W S F T L E D N N
 GCAGTGGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAGAGGATAACAAC
 396
 I F P K Q Y P I I N F T T A G A T V Q S Y T
 ATATTTCCCAACAATACCCAATTATAAACTTTACCACAGCGGGTGCCACTGTGCAAAGCTACACA
 A-chain
 N F I R A V R G R L T T G A D V R H E I P V
 AACTTTATCAGAGCTGTTGCGGGTCGTTTAACTGAGCTGATGTGAGACATGAAATACCAGTG
 519
 L P N R V G L P I N Q R F I L V E L S N H A
 TTGCCAAACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCAAATCATGCA
 594
 E L S V T L A L D V T N A Y V V G Y R A G N
 GAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATATGTGGTCGGCTACCGTGCTGGAAAT
 660
 S A Y F F H P D N Q E D A E A I T H L F T D
 AGCGCATATTTCTTTCATCCTGACAATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGAT
 726
 V Q N R Y T F A F G G N Y D R L E Q L A G N
 GTTCAAATCGATATACATTGCGCTTTGGTGGTAATTATGATAGACTGAACAACTTGCTGGTAAT
 792
 L R E N I E L G N G P L E E A I S A L Y Y Y
 CTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTTATTATTAC
 858
 S T G G T Q L P T L A R S F I I C I Q M I S
 AGTACTGGTGGCACTCAGCTTCCAACCTCTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCA
 924
 E A A R F Q Y I E G E M R T R I R Y N R R S
 GAAGCAGCAAGATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGAGATCT
 990
 A P D P S V I T L E N S W G R L S T A I Q E
 GCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGACTTTCAACTGCAATTCAAGAG
 1056
 S N Q G A F A S P I Q L Q R R N G S K F S V
 TCTAACCAAGGAGCCTTTGCTAGTCCAATTCAACTGCAAGACGTAATGGTTCCAAATTCAAGTG
 1122
 Y D V S I L I P I I A L M V Y R C A P P P S
 TACGATGTGAGTATATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCATCG
 Fsp I
 Fsp I
 Fsp I

FIG. 11-I

1188
 S Q F S L L I R P V V ^{Ban I} P N F N A D V C M D P
 TCACAGTTTTCTTTGCTTATAAGGCCAGTGGTACCAAATTTTAATGCTGATGTTTGTATGGATCCT
 A-chain Linker Peptide IB-chain
 1254
 E P I V R I V G R N G L C V D V R D G R F H
 GAGCCCATAGTGCATATCGTAGGTCGAAATGGTCTATGTGTTGATGTTAGGGATGGAAGATTCCAC
 1320
 N G N A I Q L W P C K S N T D A N Q L W T L
 AACGGAAACGCAATACAGTTGTGGCCATGCAAGTCTAATACAGATGCAATCAGCTCTGGACTTTG
 1386
 K R D N T I R S N G K C L T T Y G Y S P G V
 AAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTAACTACTTACGGGTACAGTCCGGGAGTC
 1452
 Y V M I Y D C N T A A T D A T R W Q I W D N
 TATGTGATGATCTATGATTGCAATACTGCTGCAACTGATGCCACCCGCTGGCAAATATGGGATAAT
 1518
 G T I I N P R S S L V L A A T S G N S G T T
 GGAACCATCATAAATCCCAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACA
 1584
 L T V Q T N I Y A V S Q G W L P T N N T Q P
 CTTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACTAATAATACACAACCT
 1650
 F V T T I V G L Y G L C L Q A N S G Q V W I
 TTTGTGACAACCATTTGTTGGGCTATATGGTCTGTGCTTGCAAGCAAATAGTGGACAAGTATGGATA
 1716
 E D C S S E K A E Q Q W A L Y A D G S I R P
 GAGGACTGTAGCAGTGAAAAGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCT
 1782
 Q Q N R D N C L T S D S N I R E T V V K I L
 CAGCAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGTTGTCAAGATCCTC
 1848
 S C G P A S S G Q R W M F K N D G T I L N L
 TCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGTTCAAGAATGATGGAACCATTTTAAATTTG
 1914
 Y S G L V L D V R A S D P S L K Q I I L Y P
 TATAGTGGGTTGGTGTAGATGTGAGGGCATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCT
 1980
 L H G D P N Q I W L P L F * *
 CTCCATGGTGACCCAAACCAATATGGTTACCATTATTTTGA TAGACAGATTACTCTCTTGCAGTG
 2046
 TGTATGTCCTGCCATGAAAATAGATGGCTTAAATAAAAAGGACATTGTAAATTTTGTAACTGAAAG
 2112
 GACAGCAAGTTATTGCAGTCCAGTATCTAATAAGAGCACAACTATTGTCTTGTGCATTCTAAATTT
 2178
 ATGGATGAATGTATGAATAAAGCTAATTATTTTGGTCATCAGACTTGATATCTTTTTGAATAAAAT
 2244
 AAATAATAATGTTTTTTCAAACCTATAAACTAATGAATGATATGAATATAAATGCGGAGACTAGT
 2310
 CAATCTTTTATGTAATTCTATGATGATAAAAGCTT

FIG. 11-2

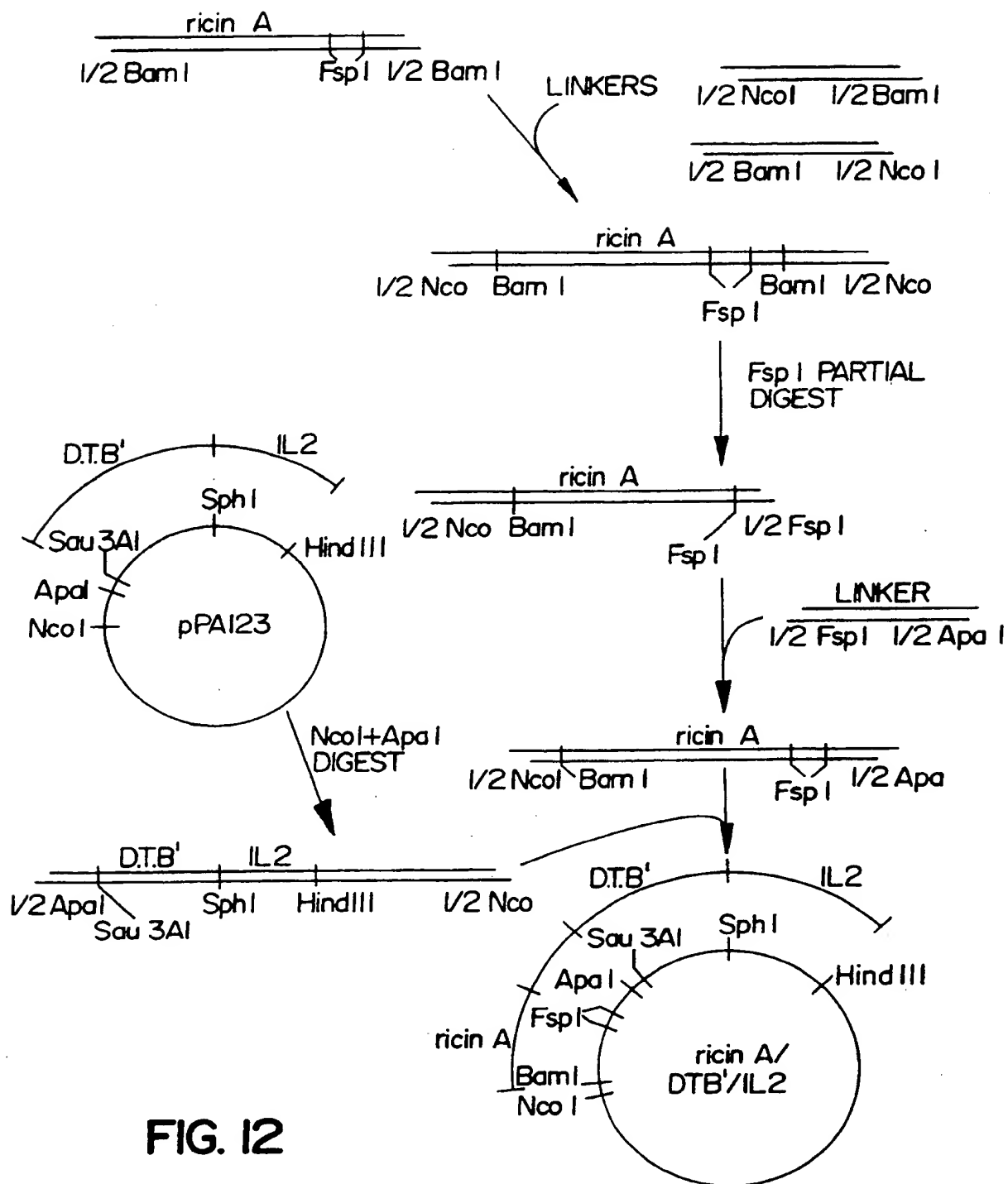


FIG. 12

CTT	CAT	CGT	CGT	15	CCA	ACT	GAC	CTT	GAG	30	TAG	TTT	CGC	GGT	45	TTT	GGG	TAT	AAG	60	TGC	CAC	CAC	CAG	TGC	75	CGG
CAG	TGT	AGT	CAG	90	TAG	TTT	GTT	GCT	GGA	105	CGT	TAC	CGC	CAA	120	GCG	TGT	TAC	CGC	135	CTA	GAT	TAG	ACT	GTG	150	GCT
GCT	GGC	GTT	GAG	165	GGA	AAC	CTG	CCT	GTA	180	GAG	GCC	CTA	AAA	195	CAG	AGA	CCT	CAC	210	TCC	CGG	GGA	GCC	AGC	225	ATG
TCC	ACT	GCG	GTC	240	CTG	GAA	AAC	CCA	GGC	255	GGC	AGG	AAA	CTC	270	GAC	TTT	GGG	CAG	285	GAA	ACA	AGC	TAT	ATT	300	GAA
Ser	Thr	Ala	Val	315	Leu	Glu	Asn	Pro	Gly	330	Gly	Arg	Lys	Leu	345	Ser	Phe	Gly	Gln	360	Glu	Thr	Ser	Tyr	Ile	375	Glu
GAC	AAC	TGC	AAT	390	Gln	Asn	Gly	Ala	Ile	405	CTG	ATC	TTC	TCA	420	AAA	GAA	GAA	GTT	435	GGT	GCA	TTG	GCC	AAA	450	GTA
Asp	Asn	Cys	Asn	465	Thr	Glu	Asn	Asp	Val	480	Leu	Thr	His	Ile	495	Leu	Glu	Glu	Val	510	Gly	Ala	Leu	Ala	Lys	525	Val
TTG	CGC	TTA	TTT	540	GAG	GAG	AAT	GAT	GTA	555	CTG	ACC	CAC	ATT	570	TCT	AGA	CCT	TCT	585	CGT	TTA	AAG	AAA	GAT	600	GAG
Leu	Arg	Leu	Phe	615	Thr	Thr	His	Leu	Asp	630	AGC	CTG	CCT	GCT	645	ACA	AAC	ATC	ATC	660	AAG	ATC	AGG	CAT	ASP	675	ASP
TAT	GAA	TTT	TTC	690	ACC	Thr	His	Leu	Asp	705	Ser	Leu	Pro	Ala	720	ACA	GTG	CCC	TGG	735	Lys	CCA	AGA	ACC	ATT	750	CAA
ATT	GGT	GCC	ACT	765	GTC	Val	His	Glu	Ser	780	GAT	AAG	AAG	Lys	795	ACA	GTG	CCC	TGG	810	585	CCA	AGA	ACC	ATT	825	Gln
Ile	Gly	Ala	Thr	840	Val	Val	His	Leu	Ser	855	AGC	TAT	GGA	GCG	870	Thr	Val	Pro	Trp	885	TTC	CTG	GAT	TTT	AAA	890	GAT
GAG	CTG	GAC	AGA	900	Phe	Arg	Ala	Asn	Gln	915	Ser	Tyr	Gly	Ala	930	Leu	Asp	Ala	Asp	945	CAC	Pro	Gly	Phe	Lys	960	ASP
CCT	GTG	TAC	CGT	975	GCA	Ala	Arg	Arg	Lys	990	GCT	GAC	ATT	GCC	1005	AAC	TAC	CGC	CAT	1020	GGG	CAG	CCC	ATC	CCT	1035	CGA
Pro	Val	Tyr	Arg	1050	Ala	Arg	Ala	Arg	Lys	1065	Ala	Asp	Ile	Ala	1080	Asn	Tyr	Arg	His	1095	Gly	Gln	Pro	Ile	Pro	1110	Arg
GTG	GAA	TAC	ATG	1125	GAG	Glu	Glu	Lys	Lys	1140	TGG	GGC	ACA	GTG	1155	AAG	ACT	CTG	AAG	1170	TCC	TTG	TAT	AAA	ACC	1185	CAT
Val	Glu	Tyr	Met	1200	Glu	Glu	Glu	Lys	Lys	1215	Trp	Gly	Thr	Val	1230	Lys	Thr	Leu	Lys	1245	Ser	Leu	Tyr	Lys	Thr	1260	His
GCT	TGC	TAT	GAG	1275	TAC	Tyr	Asn	His	Ile	1290	CTT	CTT	GAA	AAG	1305	TGT	GGC	TTC	CAT	1320	GAA	GAT	AAC	ATT	CCC	1335	CAG
Ala	Cys	Tyr	Glu	1350	Tyr	Thr	Asn	His	Ile	1365	Leu	Leu	Glu	Lys	1380	Cys	Gly	Phe	His	1395	Glu	Asp	Asn	Ile	Pro	1410	Gln

FIG. 13-1

CTG GAA GAC GTT TCT CAA TTC CTG CAG ACT TGC ACT GGT TTC CGC CTC CGA CCT GTG GCT GGC CTG CTT TCC 975	Leu Glu Asp Val Ser Gln Phe Leu Phe Leu Gln	990	915	930	945	960	975
CGG GAT TTC TTG GGT GGC CTG GGC TTC CAC TTC CAC TAC ATC AGA CAT GGA TCC AAG CCC ATG 1050	Arg Asp Phe Leu Gly Gly Phe Ala Phe Arg Val Phe His Cys Thr Gln Tyr Ile Arg His Gly Ser Pro	1005	1065	1080	1095	1110	1125
TAT ACC CCC GAA CCT GAC ATC TGC CAT TGC GGA CAT GTG CCC TTG TTT TCA GAT CGC AGC TTT GCC CAG 1125	Tyr Thr Pro Glu Pro Asp Ile Cys His Glu Leu Leu Gly His Val Pro Leu Phe Ser	1140	1155	1170	1185	1200	1215
TTT TCC CAG GAA ATT GGC CTT GCC TCT TCT GCA CCT GAT GAA TAC ATT GAA AAG CTC GCC ACA ATT TAC TGG 1275	Phe Ser Gln Glu Ile Gly Leu Ala Ser Leu Gly Ala Pro Asp Glu Tyr Ile Glu Lys Leu Ala Thr Ile Tyr Trp	1230	1245	1260	1275	1290	1305
TTT ACT GTG GAG TTT GGG CTC TGC AAA TGC CAA GGA GAC TCC ATA AAG GCA TAT GGT GCT GGT GGC CTC CTG TCA TCC TTT 1350	Phe Thr Val Glu Phe Gly Leu Cys Lys Gln Gly Asp Ser Ile Lys Ala Tyr Gly Ala	1320	1335	1350	1365	1380	1395
GGT GAA TTA CAG TAC TGC TTA TGC TCA GAG AAG CCA AAG CTT CTC CCC CTG GAG CTG GAG AAG ACA GCC ATC CAA AAT 1425	Gly Glu Leu Gln Tyr Cys Leu Ser Glu Lys Pro Lys Leu Leu Leu Glu Leu Glu Leu Glu Lys Thr Ala Ile Gln Asn	1440	1455	1470	1485	1500	1515
TAC ACT GTC ACG GAG TTC CAG CCC CTG TAT TAC GTG GCA GAG AGT TTT AAT GAT GCC AAG GAG AAA GTA AGG AAC 1575	Tyr Thr Val Thr Glu Phe Gln Pro Leu Tyr Tyr Val Ala Glu Ser Phe Asn Asp Ala	1530	1545	1560	1575	1590	1605
TTT GCT GCC ACA ATA CCT CGG CCC TTC TCA GTT CGC TAC GAC CCA TAC ACC CAA AGG ATT GAG GTC TTG GAC AAT 1650	Phe Ala Ala Thr Ile Pro Arg Pro Phe Ser Val Arg Tyr Asp Pro Tyr Thr Thr Gln Arg Ile Glu Val Leu Asp Asn	1620	1635	1650	1665	1680	1695
ACC CAG CAG CTT AAG ATT TTG GCT GAT TCC ATT AAC AGT GAA ATT GGA ATC CTT TGC AGT GCC CTC CAG AAA ATA 1725	Thr Gln Gln Leu Lys Ile Leu Ala Asp Ser Ile Asn Ser Glu Ile Gly Ile Leu Cys Ser Ala Leu Gln Lys Ile	1710	1725	1740	1755	1770	1785
AAG TAA AGC CAT GGA CAG AAT GTG GTC TGT CAG CTG TGA TGG AGA TCC TCG CAT TTC ATC AAG ATT AAT TAA 1875	Lys	1845	1860	1875	1890	1905	1920
AAA AAG TCC GAA AAG CAA ACC TTA ATT TGA AAT AAC AGC CTT AAA TCC TTT ACA AGA TGG AGA AAC AAC AAA TAA 1925		1940	1955	1970	1985	2000	2015
GTC AAA ATA ATC TGA AAT GAC AGG ATA TGA GTA CAT ACT CAA GAG CAT AAT GGT AAA TCT TTT GGG GTC ATC TTT 1975		1990	2005	2020	2035	2050	2065
GAT TTA GAG ATG ATA ATC CCA TAC TCT CAA TTG AGT TAA ATC ATC AGT AAT CTG TCG CAT TTC ATC AAG ATT AAT TAA 1985		2000	2015	2030	2045	2060	2075

FIG. 13-2

AAT	TTG	GGA	CCT	GCT	TCA	TTC	AAG	CTT	CAT	ATA	IGC	TTT	GCA	GAG	AAC	TCA	TAA	AGG	AGC	ATA	TAA	GGC	TAA	ATG	1950
TAA	AAC	ACA	AGA	CTG	TCA	TTA	GAA	TTG	AAT	TAT	IGG	GCT	TAA	TAT	AAA	TCG	TAA	CCT	ATG	AAG	TTT	ATT	TTC	TAT	2025
TTT	AGT	TAA	CTA	TGA	TTT	CAA	TTA	CTA	CTT	TGT	TAT	TGT	ACC	TAA	GTA	AAT	TTT	CTT	TAG	GTC	AGA	AGC	CCA	TTA	2100
AAA	TAG	TTA	CAA	GCA	TTG	AAC	TTC	TTT	AGT	ATT	ATA	TTA	ATA	TAA	AAA	CAT	TTT	TGT	ATG	TTT	TAT	TGT	AAT	CAT	2175
AAA	TAC	TGC	TGT	ATA	AGG	TAA	TAA	AAC	TCT	GCA	CCT	AAT	CCC	CAT	AAC	TTC	CAG	TAT	CAT	TTT	CCA	ATT	AAT	TAT	2250
CAA	GTC	TGT	TTT	GGG	AAA	CAC	TTT	GAG	GAC	ATT	TAT	GAT	GCA	GAT	GAT	GTT	GAC	TAA	AGG	CTT	GGT	TGG	TAG	ATA	2325
TTC	AGG	AAA	TGT	TCA	CTG	AAI	AAA	TAA	GTA	AAT	ACA	TTA	TTG	AAA	AGC	AAA	TCT	GTA	TAA	ATG	TGA	AAT	TTT	TAT	2400
TTG	TAT	TAG	TAA	TAA	AAC	ATT	AGT	AGT	TTA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	2445

FIG. 13-3

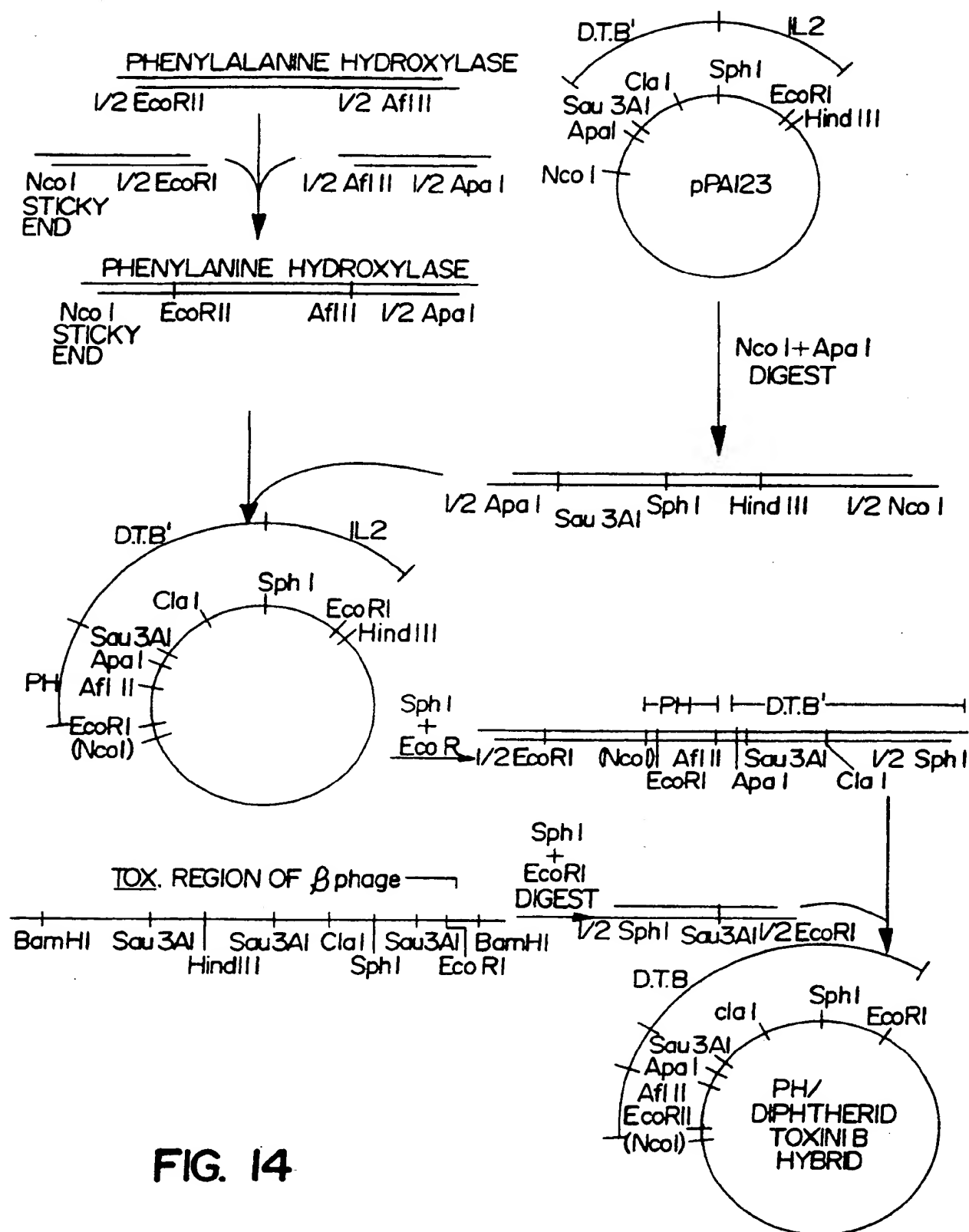


FIG. 14

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